A detailed investigation into varied aspects of the Maillard reaction: *In vitro* studies on protein crosslinking and the role of amadoriase enzymes

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Abstract

The Maillard reaction represents a complex series of processes, initiated on reaction of a carbonyl moeity with an amine. Amongst the vast array of products (advanced glycation end products, AGEs) are protein crosslinks, which can form on reaction of α-dicarbonyls with lysine and arginine residues, to form molecular bridges between proteins. It is thought that these crosslinks affect the native function of the protein, due to a structural change in the protein, and they have been implicated in the progression of some age-related diseases.

Little is known about the precise amino acid requirements for protein crosslinking. Thus, this thesis sought to determine which amino acids were critical for crosslinking to occur. A lysine-containing (arginine-free) protein and an arginine-containing (lysine-free) protein were reacted with three α-dicarbonyl compounds, methylglyoxal, glyoxal and diacetyl. It was demonstrated, for the first time *in vitro*, that an arginine residue is not essential for protein crosslinking to occur. Only an *N*-terminus or a lysine residue is required for crosslinking with the three α-dicarbonyls tested.

A model protein, RNase A, which contains both lysine and arginine, was also incubated with the three dicarbonyls. Parallel measurements on crosslinking and activity were performed in order to test the assumption that crosslinking affects the function of the protein. To establish whether the loss in function was a direct result of the crosslinking process, or a co-incident event, two previously reported crosslinking inhibitors, aminoguanidine and 3,5-dimethylpyrazole-1-carboxamidine, were included. The results demonstrated that although crosslinking of RNase A could be inhibited by these compounds in the presence of α-dicarbonyl, the activity of RNase A was not necessarily preserved. Inhibition of crosslinking may not preserve the function of the protein, as evidenced by the RNase A incubated with methylglyoxal and 3,5-dimethylpyrazole-1-carboxamidine. This loss in activity but inhibition of crosslinking may be explained by the presence of non-crosslinking AGEs that have formed on the protein, rendering it inactive. These results are significant when considering any approach to intervention of the Maillard reaction.

Also examined was a recently-discovered enzyme, amadoriase I, which has been shown to reverse the early stages of the Maillard reaction under some experimental conditions. A time-resolved assay was developed that allowed for accurate determination of kinetic parameters and purification of amadoriase I. By this method, amadoriase I was shown to have a K_m of 11 μM for fructosyl propylamine, a known substrate, and a k_{cat}/K_m of 3.25×10^5, both of which differ from previous literature reports. This assay was also used to successfully measure the activity of two mutants, H357N and S370A, which were created in order to determine which amino acid residues within amadoriase I are critical for catalysis. Both mutants were catalytically inactive, demonstrating their importance in amadoriase I.

Finally, a second assay was developed in order to determine whether amadoriase I could act on a mildly glycated protein. Amadoriase I is reported not to turnover glycated protein, the physiologically relevant substrate. However, past experiments have involved conditions that favour formation of a heavily glycated protein, which may be inaccessible to amadoriase I. The novel assay method involved direct assessment of activity of the protein substrate, before and after treatment with amadoriase I. The results confirmed previous studies that amadoriase I could not use glycated protein as a substrate, even in the case of a mildly glycated protein. The assay method was also employed to search for a potential amadoriase enzyme from a Pseudomonad which had been isolated from raw milk. The crude extract, however, did not show any deglycating activity as assessed by this assay and the time-resolved assay.

The studies undertaken in this thesis will inform design of therapeutic strategies aimed at inhibiting or undoing the damaging effects of Maillard chemistry *in vivo*.

Acknowledgements

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Finally, to Carl, it's pretty difficult to summarise how important you've been in helping me through in a sentence. Here goes: thanks for your wonderful support, humour, generosity, ability to make an average day great, and having more questions than answers.

Abbreviations

ΔA_{414}	Change in absorbance at 414 nm
A_{340}	Absorbance at 340 nm
A_{420}	Absorbance at 420 nm
A_{595}	Absorbance at 595 nm
ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
ALE	Advanced lipoxidation end product
Amp^r	Ampicillin resistance
ANOVA	Analysis of variance
A.U.	Absorbance units
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
°C	Degrees Celsius
Cam^r	Chloramphenicol resistance
CEL	N^{ε}-(carboxyethyl)lysine
CML	N^{ε}-(carboxymethyl)lysine
Da	Dalton
ddH_2O	Doubly distilled water
df	Degrees of freedom
DGAP	D-glyceraldehyde-3-phosphate
DHAP	Dihydroxyacetone-phosphate
dH_2O	Distilled water
DMF	Dimethylformamide
DMPC	3,5-Dimethylpyrazole-1-carboxamidine
DMSO	Dimethyl sulfoxide
EAGLE	Either advanced glycation or lipoxidation end products
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact ionisation
EtOH	Ethanol
FAD	Flavin adenine dinucleotide

FC/fc	Frozen control
G	Gram
g	Gravity
GC	Gas chromatography
GODIC	2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate
GOLA	N6-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine
GOLD	Glyoxal lysine dimer
HBH	Hydroxybenzoic acid hydrazide
HPLC	High performance liquid chromatography
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
h	Hours
IC/ic	Incubated control
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
k_{cat}	Catalytic constant / turnover number
K_d	Dissociation constant
kDa	Kilodalton
K_m	Michaelis constant
L	Litre
LB	Luria-Bertani media
mg	Milligram
min	Minute
mL	Millilitres
mM	Millimolar
M	Molar
MODIC	2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene} amino)hexanoate
MOLD	Methylglyoxal lysine dimer
MOPS	4-Morpholinepropanesulfonic acid

MS	Mean square
MW	Molecular weight
MWCO	Molecular weight cutoff
nM	Nanomolar
nm	Nanometres
NA	Nutrient agar
NMR	Nuclear magnetic resonance
o	*Ortho*
O/N	Overnight
*o*PA	*o*-Phthaldialdehyde
*o*PDA	*o*-Phenylenediamine
p	*Para*
P	Probability statistic
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNase A	Ribonuclease A
rpm	Revolutions per minute
RST	Renin substrate tetradecapeptide
RT	Room temperature
sdH_2O	Sterile distilled water
sec	Second
sem	Standard error of the mean
SDS	Sodium dodecyl sulfate
TAE	Tris EDTA electrophoresis buffer
TE	Tris EDTA buffer
TIM	Triose phosphate isomerase
µg	Microgram
µL	Microlitre
µM	Micromolar
UV	Ultra violet
V_{max}	Maximal velocity (limit)
V	Volt

vol	Volume
w/v	Weight per volume
xlink	Crosslink

Chapter One

Introduction

1.1 Background

The Maillard reaction encompasses a complex network of non-enzymatic reactions that are initiated by the condensation of an amine with a carbonyl compound. A multitude of products can be formed during this process. Due to the ubiquitous nature of both starting materials, the Maillard reaction has far-reaching consequences, from food chemistry to involvement in medical science (1).

Proteins contain a number of amines, for example, the *N*-terminus, lysine and arginine residues, which provide sites for the covalent attachment of sugars. This process of attachment, termed protein glycation, leads to a change in protein structure, which can in turn affect protein function (2-4).

One specific type of glycation results in the formation of protein crosslinks. These crosslinks are generated *via* bridging of the protein-bound amines by carbonyl containing compounds. Protein crosslinks are thought to result in the impairment or loss of the native activity of the protein (5). However, surprisingly little is know about which amino acids are required for crosslinking to occur. Moreover, although protein crosslinking is assumed to have a deleterious effect on protein function, this has not yet been rigorously tested. The work detailed in this thesis sought to test this assumption and identify the structural requirements for protein crosslinking. The results described will be significant for future studies on attenuation of the Maillard reaction.

1.2 Initial stages of the Maillard reaction

1.2.1 Classical descriptions of the Maillard reaction

Since its discovery early last century (6), our knowledge of the mechanisms involved in the Maillard reaction has steadily increased. The initial step involves reaction of a carbonyl compound with a free amine, which appears upon first inspection seems to be a relatively simple event. To date, however, a complete reaction scheme cannot be presented (7).

L. C. Maillard pioneered work in this area in 1912, with the observation that on heating sugars with amino acids, a yellow-brown colour developed (6,7). Elucidation of the chemical pathways underlying this browning phenomenon was undertaken by Amadori (8), Kuhn and Weygand (9), Simon and Kraus (10), Heyns *et al.* (11), and Hodge and Rist (12). This culminated in the publication of the "classical" pathway, which is also referred to as the Hodge pathway (Figure 1.1) (1,12). The first step involves the nucleophilic attack of an amine at the carbonyl carbon. The reaction product is subsequently dehydrated to form an imine (Schiff base) (Figure 1.1). Studies have shown that the imine generally exists as a cyclic glycosylamine (13). The labile imine then tautomerises to the enaminol, which can undergo further reaction to form either the stable Amadori product (from reaction of an aldose sugar) or Heyns product (from reaction of a ketose sugar) (Figure 1.1) (1).

The availability of amines within proteins for modification by carbonyl compounds means that the Maillard reaction is biologically relevant. Indeed, it has been suggested that the Maillard reaction is involved in the pathophysiology of some diseases, such as cataract formation (2,14). In the early stages of Maillard research from a medical perspective, the reactive carbonyl compounds studied were reducing pentose and hexose sugars (14,15). This was mainly due to the influence of food chemistry, where studies generally focused on these sugars. More recently, the scope has broadened to include α-dicarbonyls (4), ascorbate and its oxidation product dehydroascorbate (16), polyol and glycolytic pathway (17) and lipid oxidation products (18). Some of these compounds result in reaction pathways that diverge from the classical route (section 1.4).

Physiological amines that can participate in the Maillard reaction include nucleotides, aminophospholipids, and protein (1,19,20). Proteins are rich in substituents such as lysine and arginine residues, which contain amine moieties in their side chains that are modified during the Maillard reaction (21,22). Histidine, proline and tryptophan contain secondary and/or tertiary amines which can participate in this chemistry but are less reactive (1,23,24).

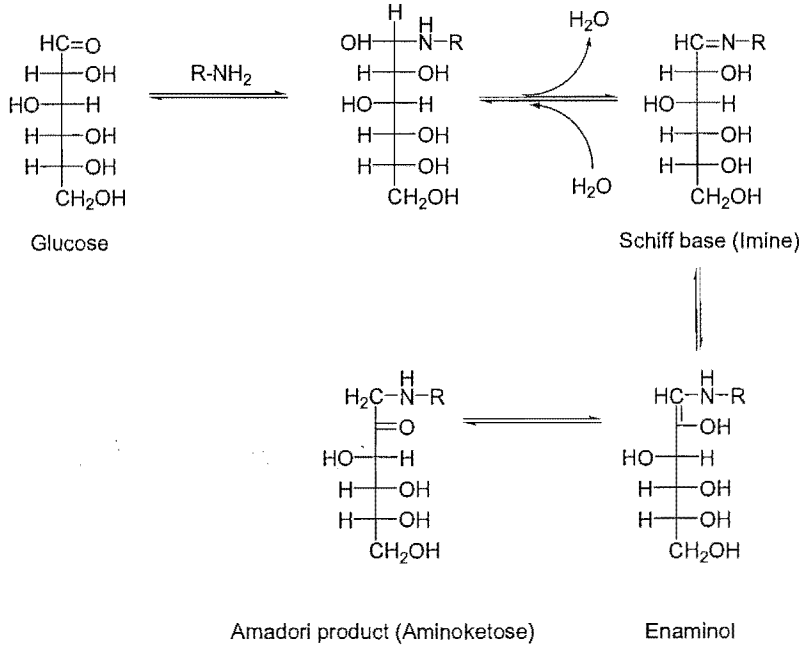


Figure 1.1: The "classical" pathway describing the initial stages of the Maillard reaction, where glucose reacts with an amine to form an Amadori product. R represents the remaining amine skeleton, *e.g.* remainder of lysine $-(CH_2)_4CH(NH_2)CO_2H$. Acyclic forms of the sugar and derivatives have been used for clarity. From Ledl *et al.* (1).

After formation of the Amadori or Heyns product, the mechanistic details of the Maillard reaction are not as well-defined. There is a vast range of reactions such as cyclisation, oxidation and condensation that can occur. These processes result in the generation of a complex array of compounds dubbed advanced glycation end products (AGEs) (25).

1.3 Later stages of the Maillard reaction – formation of advanced glycation end products (AGEs)

A number of AGEs have been isolated and identified in human tissue, where they are though to have deleterious consequences for the target protein (Figure 1.2) (26-29).

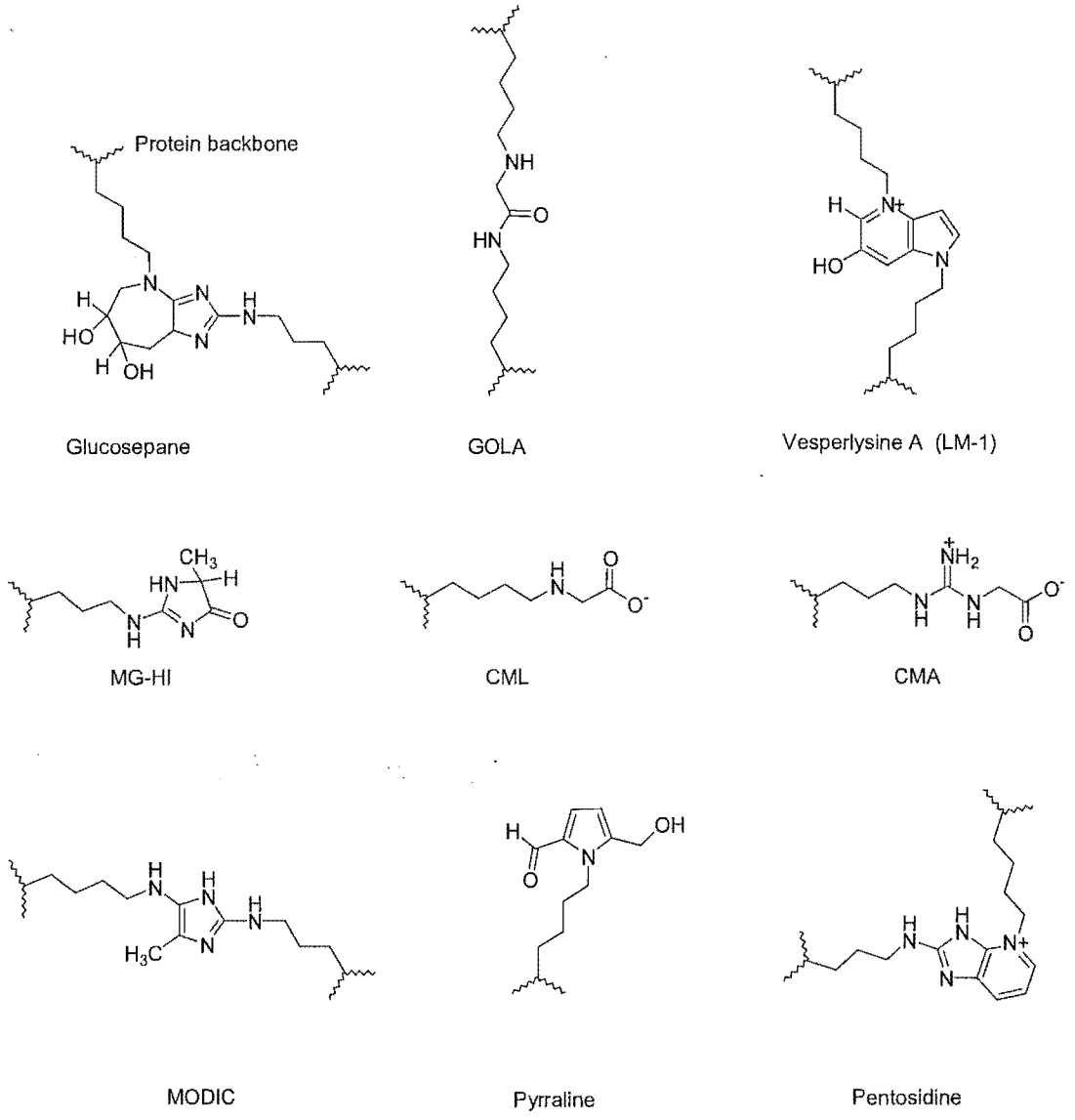


Figure 1.2: A selection of advanced glycation end products from the literature (15,30-37). Abbreviations: GOLA, N^{δ}-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine; CML N^{ε}-carboxymethyl-lysine; CMA, N^{ω}–carboxymethylarginine; MODIC, 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino)hexanoate; MG-H1, N^{δ}-(5-hydro-5-methyl-imidazolon-2-yl)-ornithine.

Those structures detailed in Figure 1.2, excluding the remainder of the protein, are relatively small in size compared to a class of AGEs or Maillard reaction products, the melanoidins. These are generally restricted to food systems, where they are thought to contribute to a class of high molecular weight coloured products and exist at molecular weights up to 10 kDa (38,39).

Although a number of AGEs have been identified *in vitro* and subsequently isolated *ex vivo*, many remain uncharacterised (27). As our knowledge increases in this area, it becomes evident that conventional methods undertaken to isolate AGEs may not be suitable for the isolation of other, as yet undefined, products. Past studies have exploited properties of AGEs that have made them amenable to isolation, for example, the fluorescent properties of pentosidine (37). Following successful isolation, the product can be identified by NMR and mass spectrometry. However, some suggest that major AGEs are non-fluorescent and non-ultraviolet active, presenting a significant barrier in the search for novel AGEs (40).

Problems with characterisation of AGEs can also arise from the environment in which the AGEs are isolated. Model systems involving proteins may result in the formation of high molecular weight insoluble material, and isolation of the AGE of interest can prove difficult. Previously, isolation of AGEs generally involved treatment of the glycated protein with 6 M acid digest (26,37). This leads to the selection of only acid-stable AGEs and, possibly, to the generation of artifactual products that have been modified by the work-up procedures (32). However, advances in methodology, such as enzymatic digests in place of acid, have meant that some of these previously-undetectable crosslinks have been elucidated (31).

1.4 Deviation from the Hodge pathway

As more has become known about Maillard chemistry, it becomes evident that more than one pathway is in operation in the early stages of the reaction. These discoveries have aided our understanding of AGE formation, since an AGE can be formed *via* a number of differing routes.

1.4.1 The contribution of glucose autooxidation products to the Maillard reaction

The elucidation of a pathway for formation of α-ketoaldehydes from autooxidation of glucose led to the proposal that these α-ketoaldehydes may participate in the Maillard reaction. This was confirmed by Wolff and Dean, who showed that α-ketoaldehydes, formed on glucose autooxidation, contributed to protein glycation (41). This work

was extended by Wells-Knecht *et al.* and Thornalley *et al.* who described the formation of glyoxal and methylglyoxal from glucose *in vitro* (42,43) (Figure 1.3).

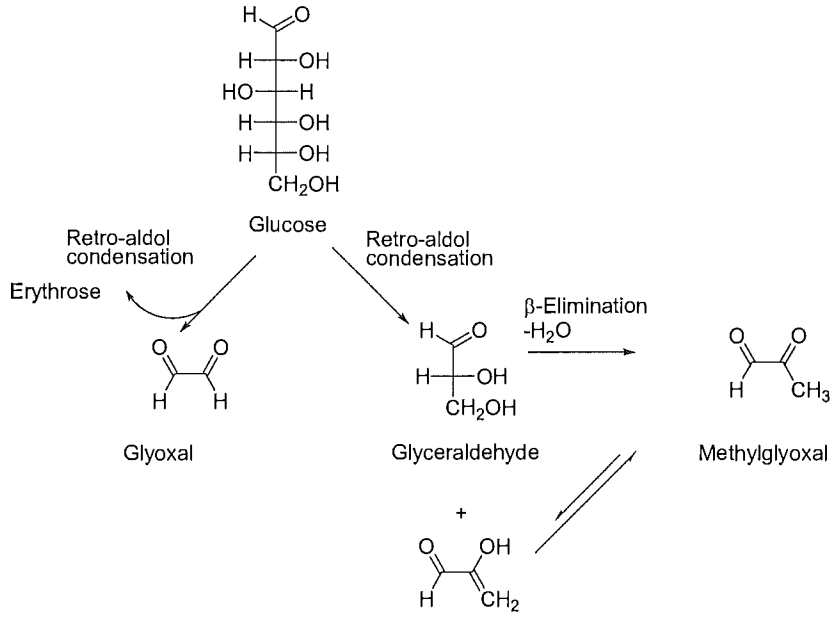


Figure 1.3: Formation of glyoxal and methylglyoxal by autooxidation of glucose (42,43).

This new pathway resulted in a re-evaluation of the Maillard reaction as, until this point, the Amadori or Heyns product was the only known route to AGE formation. It is now evident that a number of AGEs can be formed from methylglyoxal and glyoxal (25,42,44) (Figure 1.4). These products are generally dubbed advanced glycoxidation end products (42).

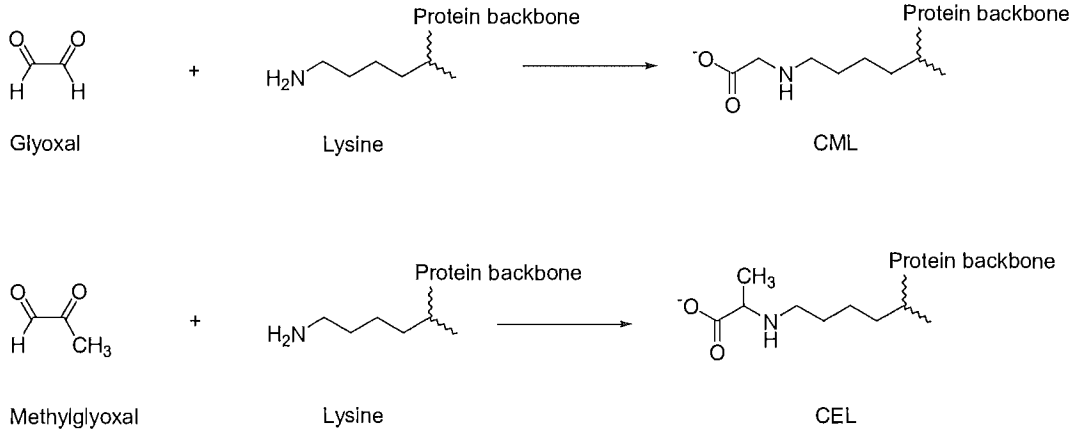


Figure 1.4: Two AGEs formed on autooxidation of glucose. Abbreviation: CEL, N^{ε}-carboxyethyl-lysine; CML, N^{ε}-carboxymethyl-lysine (42,44).

1.4.2 The Namiki pathway

The Namiki pathway describes retro-aldol condensation of the Schiff base adduct and has been shown to yield glyoxal (45) (Figure 1.5). Further reaction of these products can result in the formation of the AGE N^{ε}-carboxymethyl-lysine (CML) and protein crosslinks (Figure 1.5) (45). It has also been reported that the major source of one dicarbonyl formed in glucose-amine model systems, glyoxal, is neither through autooxidation of glucose nor the break-down of the Amadori product, but through the Namiki pathway (45).

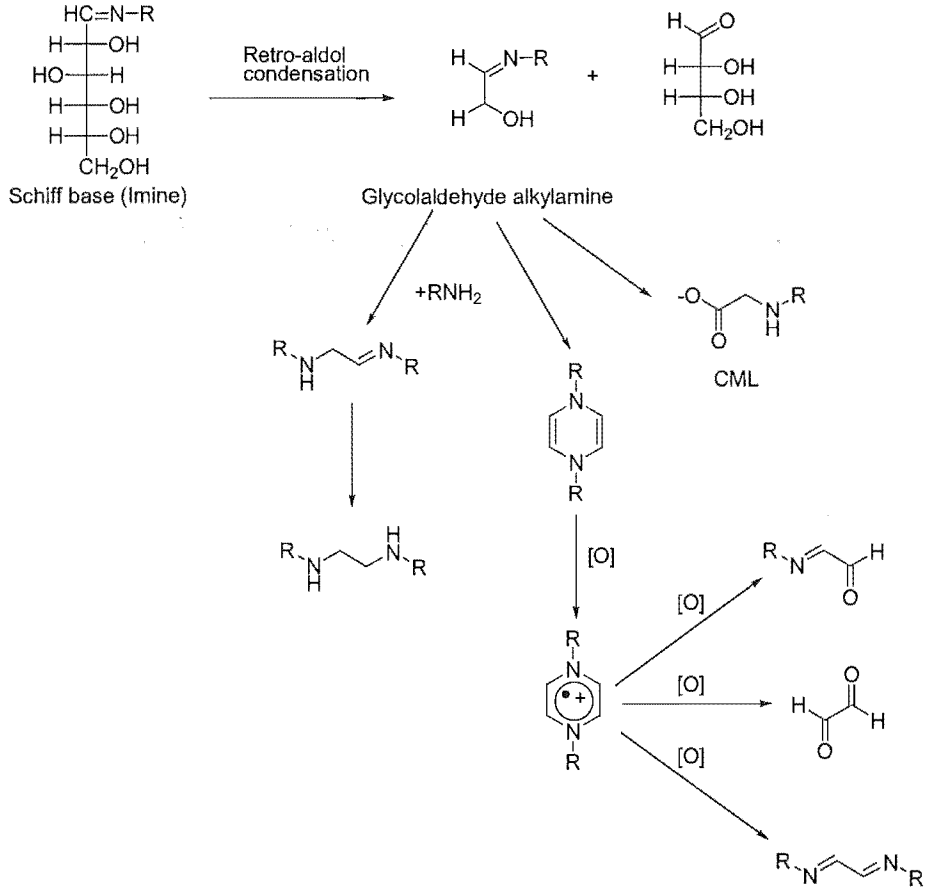


Figure 1.5: Proposed reaction scheme of the Namiki pathway involving the retro-aldol condensation of the Schiff base (imine) and its reaction products. After Glomb and Monnier (45). R represents the remainder of the amine skeleton.

1.4.3 The role of Michael additions in the Maillard reaction

Another pathway within the Maillard reaction involves the reaction of lipid peroxidation products to yield advanced lipoxidation products (ALEs), similar to AGEs. The formation of some of these products is thought to involve differing chemistry to that discussed thus far, with the formation of adducts *via* Michael addition of lysine residues to lipid peroxidation products, such as 4-hydroxynonenal (HNE) and crotonaldehyde (Figure 1.6) (23,46,47).

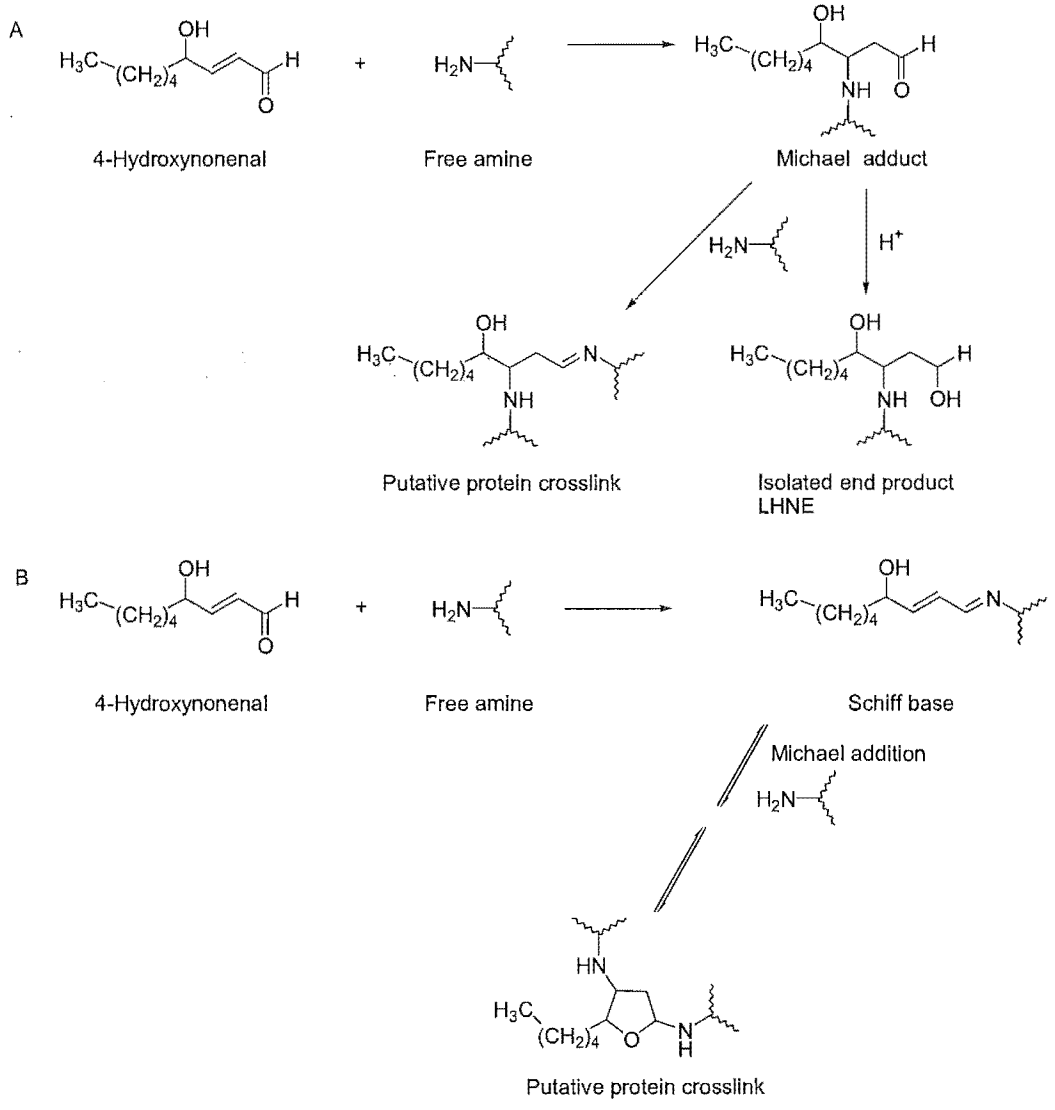


Figure 1.6: Proposed pathways on reaction of a lipid peroxidation product with an amine *via* A, Michael addition (and subsequent Schiff base formation in some cases), (48) or B, Schiff base formation followed by Michael addition (49). Abbreviation: LHNE, 3-(N^{ε}- lysino)-4-hydroxynonan-1-ol. After Uchida *et al.* (50).

There are two routes to the formation of end products of the reaction on incubation of protein with these lipid peroxidation products: Michael addition and Schiff base formation (Figure 1.6) (23). Some suggest that after initial Michael addition of the side chain of amino acids such as lysine, cysteine or histidine to the target α,β–unsaturated carbonyl compound (such as HNE), a secondary reaction can occur that will involve reaction of a protein-bound amine with the free C1 carbonyl to yield a Schiff base (Figure 1.6 A) (48,50). Others have suggested that the formation of the Schiff base pre-empts the Michael addition, and that the isolated Schiff base Michael adducts, formed on reaction of amine with HNE, can be completely reversed (Figure 1.6 B) (49,51,52).

1.4.4 Strecker degradation

The Strecker degradation appears to be confined to the area of food chemistry, where Maillard chemistry plays an important role. Strecker degradation involves degradation of α-amino acids on reaction with carbonyl compounds, ranging from glucose to α-dicarbonyls, to yield an aldehyde with one carbon fewer than the α-amino acid (Figure 1.7) (1). Studies in this area have been generally restricted to free amines, not those that are protein-bound (53,54). However, oxidative deamidation of protein-bound lysine residues has been reported and is thought to occur *via* a Strecker-type reaction (55).

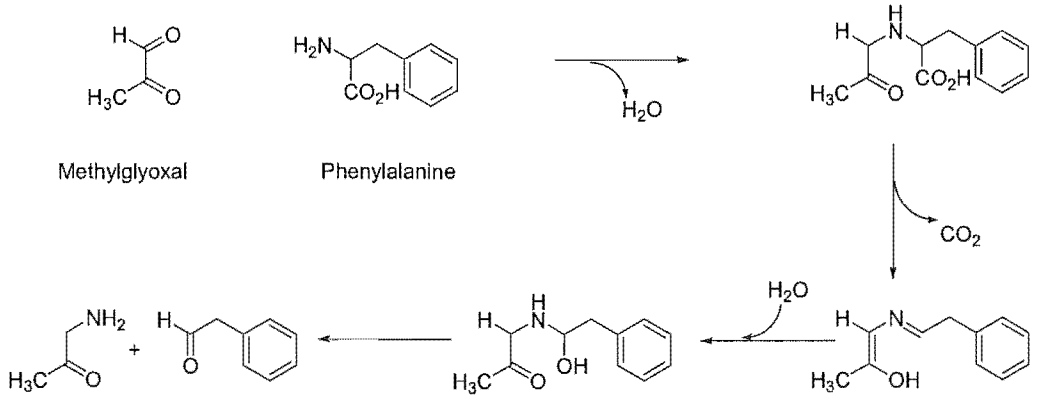


Figure 1.7: Strecker degradation of phenylalanine on reaction with methylglyoxal (53).

1.4.5 Which pathways are of most physiological importance?

There are a number of differing pathways that can lead to the same product. The formation of CML is used here as an illustrative case study. *In vitro* studies have shown that CML can form in a number of ways. The formation of CML was first reported by Ahmed *et al.*, who suggested that CML was to generated solely from the reaction of glucose with an amine *via* metal-catalysed break-down of the Amadori product (Figure 1.8) (15, 56). However, following the discovery that glucose autooxidation could result in glyoxal, a route to CML formation from the reaction of glyoxal with lysine was proposed. Formation of CML was thought to occur *via* either Cannizarro rearrangement or enolisation, dehydration and elimination reactions, of the Schiff base adduct formed on reaction of lysine with glyoxal (Figure 1.8) (42). In the same year, CML was shown to form through degradation of a glucose-lysine Schiff base (Figure 1.8) (45).

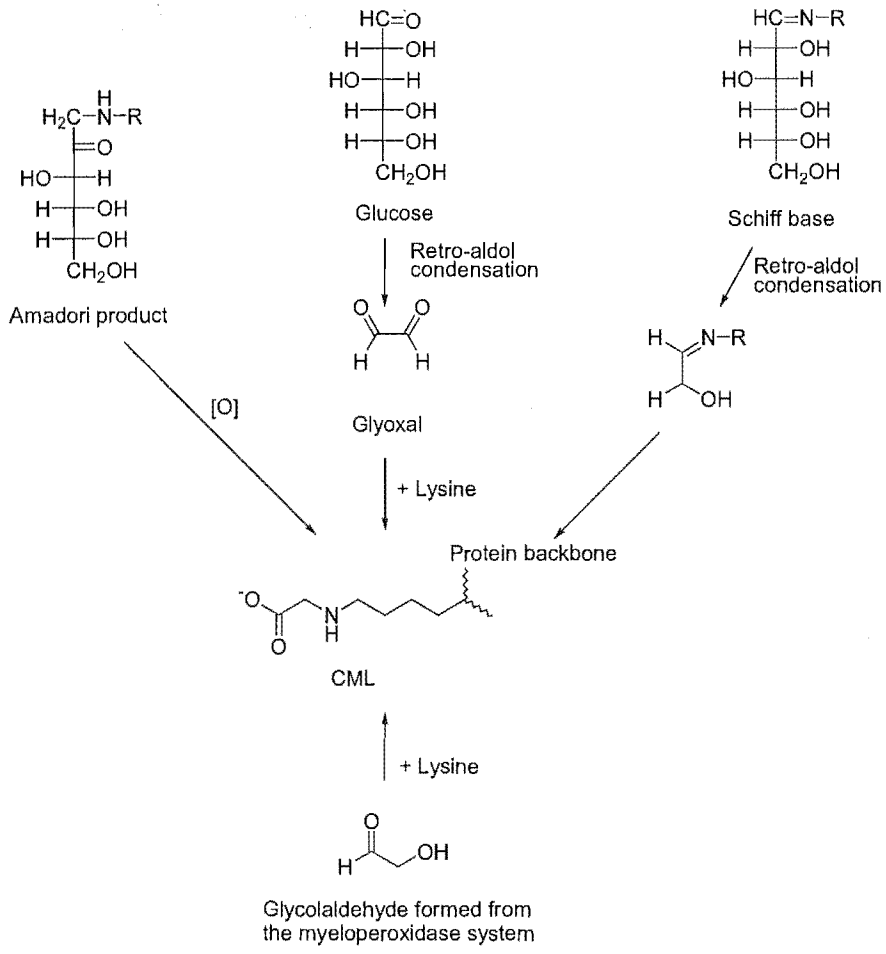


Figure 1.8: Current pathways to the formation of CML *in vivo* (15,42,45,57).

The same work also reported that, the degradation of the Amadori product generated on reaction of glucose with lysine resulted in the formation of CML (45). The authors suggested that as there is a significant concentration of Amadori product in human tissue, the degradation of the Amadori product is the most physiologically significant route to CML formation, followed by formation *via* fragmentation of the Schiff base adduct. Both of these pathways to CML were proposed to be very much more significant than that involving the autooxidation of glucose (45).

CML has also been identified on reaction of protein with hypochlorous acid (an initial product of the myeloperoxidase system) and serine, which is an excellent substrate for the reactions in the myeloperoxidase pathway (Figure 1.8) (57). Due to the fact that CML can be formed by lipoxidation products and through reaction of α-dicarbonyls, this product can be ascribed the acronym EAGLE (either advanced glycation or lipoxidation end product) (25). As more becomes known about the later stages of the Maillard reaction, many compounds that were traditionally referred to as AGEs now fall into the EAGLE category, including CML (25). Mathematical modeling based on existing *in vitro* studies has suggested that the route to CML *via* glyoxal, formed through the autooxidation of glucose, is of significant importance although the myeloperoxidase system is not considered (58). This conflicts with the earlier results from studies by Glomb and Monnier (45).

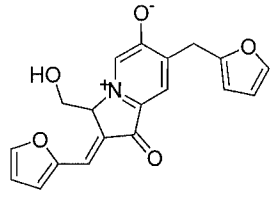
1.5 Significance of the Maillard reaction

The competing pathways outlined in the above sections result in a number of products which can have a dramatic effect in two quite different disciplines, food science and medical science, which will be discussed in turn below.

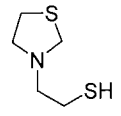
1.5.1 Implications for the food industry

Preparation of foods can involve the presence of both starting materials, amine (such as protein or supplemented amino acids) and carbonyls (such as glucose). Food processing conditions can include high temperature and pressure (7), which result in the formation of many Maillard reaction products that can vary in concentration under differing reaction conditions (59). Many changes in the properties of food have been

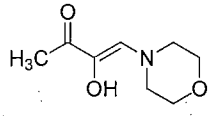
attributed to the Maillard reaction such as browning, production of flavour and aroma compounds, bioactive compounds, changes in texture and a decrease in nutritional quality (7). Some of these provide a positive effect, whilst others have a negative effect. Figure 1.9 depicts a selection of Maillard products that are thought to contribute to some of the above phenomena, which were isolated under food processing conditions.



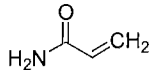
1-Oxo-2,3-dihydro-1H-indolizinium-6-olate
Bitter tasting



N-(2-Mercaptoethyl)1,3-thiazolidine
Popcorn-like aroma



3-Hydroxy-4-(morpholino)-3-buten-2-one
Antioxidant



Acrylamide
Carcinogen

Figure 1.9: A selection of Maillard reaction products that have been isolated under food processing conditions (60-63).

1.5.2 Implications for medical science

Until the 1970s, studies of the Maillard reaction were limited to the area of food chemistry. However, in the last 30 years, the formation of AGEs under physiological conditions has been extensively investigated. The focus of this research has broadened to include their role in the pathophysiology of diseases, such as diabetes, cataract formation and Alzheimer's.

Current hypotheses

Work in this area has resulted in the development of the hypothesis that AGEs that form *in vivo* can result in deleterious modification of structural proteins, enzymes, lipoproteins and DNA. These AGEs are thought to effect a change in structure, and thus function, of the target biomolecule (25). These modifications are implicated in some of the complications observed in diabetics, such as cataract formation (14).

This has been extended with the glycoxidation hypothesis, where oxidative stress is thought to play an important role in the formation of AGEs (64). An example of where this is important is the formation of the reactive α-dicarbonyls, methylglyoxal and glyoxal from glucose, which have been shown to be reactive participants in the Maillard reaction (section 1.4.1). The lipoxidation hypothesis is also beginning to gain more attention. It proposes that products of lipid oxidation, such as 4-hydroxynonenal, can react with proteins *via* lysine and histidine, to form advanced lipoxidation end products (ALEs) (section 1.4.3) (23,48).

Contribution of AGEs to the pathophysiology of diseases

Those researchers with a focus on the medical aspects of the Maillard reaction generally study age-related diseases such as cataract formation (2), Alzheimer's (65), and diabetes (66), particularly the latter due to the fact that this condition results in poorly regulated glucose levels. Simply aging also appears to result in a buildup of Maillard reaction products (67), albeit to a lesser extent than in those with diabetes (68). This is particularly true in long-lived proteins, such as collagen, where AGEs can build up due to the slow turnover of the protein (69).

The eye lens contains a number of long-lived proteins including the α-crystallins. α-Crystallins constitute a large proportion of the eye lens and are susceptible to modification *via* the Maillard reaction (2,70,71). Some of the AGEs formed on modification of α-crystallins are thought to play a role in cataract formation (72). The maintenance of the native protein structure is critical for the transparency of the eye lens. Modification in structure, and therefore organisation, within the lens results in light scattering and reduced transparency (73). It is thought that glycation could effect such a change in the structural organization of the eye lens (2). The AGEs outlined in Figure 1.10, isolated from human eye lens α-crystallins, are shown to be elevated as a result of ageing (27), and their concentration was further increased in patients with cataracts (27,74,75).

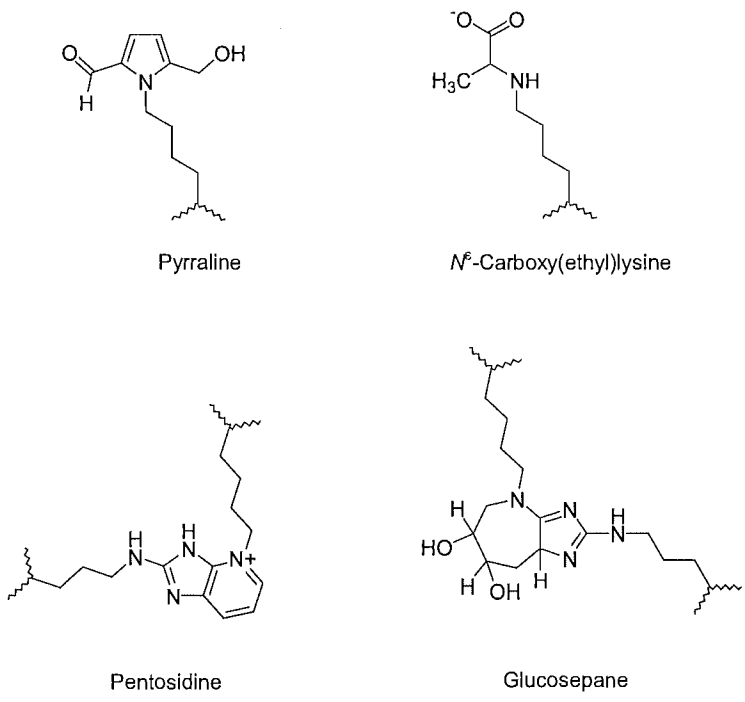


Figure 1.10: A selection of AGEs that have been isolated in lens tissue (36,37,75,76).

Perturbation of cellular function by AGEs

The effects of AGEs have so far focused on their direct impact on structural proteins. However, researchers have also examined modulation of cellular function following interaction of AGEs with cell surface binding sites. An early report in this area showed that AGEs caused a range of disturbances in cellular function (77). Attempts to isolate the protein that facilitated the entry of AGEs into the target cell resulted in the isolation of a 35 kDa polypeptide, termed receptor for advanced glycosylation end products (RAGE) (77,78). Some have hypothesised that this receptor, along with other receptors known to bind AGEs, are "accidental receptors" in that they have the capacity to bind other ligands more efficiently than AGE-modified proteins. This is demonstrated by the RAGE receptor which binds amphoterin, a protein related to the metastasis of tumours, with a higher affinity than AGEs (79). Interestingly, RAGE has been shown to bind amyloid-β-peptide, resulting in enhanced neural toxicity, and RAGE expression is increased in affected neurons in the Alzheimer's brain (80,81). RAGE is also thought to activate the cellular cascade involving $p21^{ras}$ and MAP kinase and modulate gene expression through NF-κB (82,83). Until recently, it was not known which particular AGEs were recognized by the RAGE receptor, only that it recognized AGE-modified proteins, which represent a heterogeneous mixture of AGEs. Work by Kislinger *et al.* has shown that CML can bind to RAGE, both *in*

vitro and *in vivo*, with activation of cell signaling pathways resulting in modulation of gene expression (84).

Other receptors reported to bind AGE-modified protein are: the macrophage scavenger receptor (MSR), the receptor complex of OST-48, 80K-H and galectin-3 (66,85). It is thought that these receptors contribute to clearing of AGEs from the cell (66). Indeed, it has been suggested by some workers that the MSR receptor on the macrophage acts to endocytose AGE-modified protein, and, with the aid of the insulin signaling pathway, results in effective elimination of AGE-protein (66,86).

1.6 Protein crosslinking *via* the Maillard reaction *in situ* and *in vivo*.

As the previous sections have outlined, the products of the Maillard reaction are many and varied. However, the remainder of this chapter, and thesis, will focus on protein crosslinks. Although some proteins are dependent on crosslinking processes for function, such as collagen (87), those involving the Maillard reaction are generally regarded as unfavourable. The vast majority of the crosslinks isolated to date involve lysine and arginine, and a more detailed summary of this literature will be presented in Chapter three. To date, there has not been a systematic study of the amino acid requirements for protein crosslinking. Therefore, this thesis aimed to assess the contribution of lysine and arginine in the crosslinking process by careful selection of well-defined starting materials. This information is valuable for designing therapeutic strategies for many diseases associated with Maillard crosslinks, including the following.

1.6.1 Diabetes

Diabetes is characterised by the lack of glucose regulation due to a deficiency in the secretion, or action, of insulin-the hormone that stimulates glucose uptake by body tissues (88). Poor control of diabetes can lead to high concentrations of glucose that may be available for participation in the Maillard reaction. Comparative studies with healthy humans have shown that crosslinks such as methylglyoxal lysine dimer (MOLD) and glyoxal lysine dimer (GOLD) are increased in some tissues of diabetic patients (89). There are a number of complications that arise during diabetes, such as

neuropathy (impairment of sensory and motor nerves), uremia (a buildup of urea in the bloodstream), retinopathy (disease of the retina) and angiopathy (disease of blood vessels). Protein crosslinking has been implicated in at least some of these diabetic complications, due to their ability to induce tissue damage and increase stiffness of structural proteins, impairing their function (5,90,91). Interestingly, some crosslink breakers have been reported to reverse some of the above effects (92,93), including reducing cardiac tissue stiffness with a concomitant increase in cardiac function (92).

1.6.2 Alzheimer's disease

The formation of the aggregates that are characteristic of Alzheimer's disease is thought to be exacerbated by the Maillard reaction. Work by Smith *et al.* detected the presence of the protein crosslink pentosidine in neurofibrillary tangle and senile plaques of Alzheimer's disease patients by immunocytochemistry (65). This is supported by another study that reported an elevated level of AGEs in Alzhemier's patients over those that did not have the disease (94). Finally, the polymerisation of the major protein in amyloid deposits, β-amyloid protein, has been shown to be accelerated by AGE-mediated crosslinking (95).

1.6.3 The aging process

As some proteins are slower to turn over than others, they are prone to glycation. Over time, these long lived proteins can become crosslinked as part of the aging process. Collagen is a good example, where pentosidine has been shown to increase with age (37,67). It is proposed that accumulation of these crosslinks results in increasing stiffness of collagen and impairment of its function (96). *In vitro* studies have shown a concomitant increase of insolubility of collagen with crosslinking (97).

1.6.4 Cataract formation

The earliest studies into the effect that crosslinking has on a protein were on the α-crystallin proteins within the eye, as discussed in section 1.5.2. The protein crosslinks MOLD and GOLD, formed on reaction of methylglyoxal and glyoxal respectively,

with α-crystallin, were found to exist in the human lens and increase with time (26). Marked elevation in protein crosslinks in the lens are thought to be due to aging and are reported to be exacerbated in diabetics (98).

1.6.5 The nature of crosslinks isolated thus far

Although a range of crosslinks has been elucidated, it is thought that the majority remain undefined (40,99). This has been due to the limitations of the techniques developed to isolated protein crosslinks, as previously discussed in section 1.3. Traditionally, isolation of these crosslinks has involved the treatment of glycated protein with concentrated acid and prolonged reflux at high temperatures (26,37). This process creates an immediate bias for selection of acid-stable crosslinks and can also result in modification of the crosslink during the isolation process. However, with methodological advances, the structures of a number of acid-labile crosslinks have been elucidated (31,99). These new methods generally involve digesting the protein with enzymes, with subsequent analysis of the fragments by LC/MS, allowing for the elucidation of crosslinks that are colourless and non-fluorescent, such as glucosepane (Figure 1.10). Immunochemistry has also played a major role in the isolation and elucidation of acid-labile crosslinks (100). Some argue that quantifying AGEs *via* immunochemistry leads to difficulties, due to factors such as uncertainty of the specificity of the antibodies used (101). Although the process of synthesising possible AGEs *in vitro* and then searching for this molecule in ageing tissue has resulted in the isolation of a number of crosslinks, it still does not provide a total picture, because some of these isolated AGEs are physiologically unstable (27).

1.6.6 Which carbonyl–containing molecules are effective at crosslinking?

A number of carbonyl-containing molecules can participate in the Maillard reaction, from α-dicarbonyl compounds that can form on the break-down of sugars (*e.g.* glyoxal formation from retro-aldol condensation of glucose, section 1.4.1) to lipid oxidation products, such as 4-hydroxynonenal (section 1.4.3). There does not appear to be any strict requirements for a successful crosslinking carbonyl, although this has not been fully assessed and is thus examined in Chapter three.

Of particular interest over the last decade has been the α-dicarbonyl compounds methylglyoxal and glyoxal, which seem more physiologically relevant to the crosslinking process than glucose. These two molecules, along with the structurally related compound, diacetyl, were selected for work in this thesis, due to their implicated role in physiological crosslinking reactions (Figure 1.11). As these compounds play a critical role in this thesis, a brief overview of these molecules in terms of their formation, catabolism and significance in the Maillard reaction is given below.

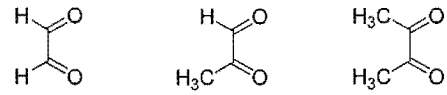


Figure 1.11: The structure of the three α-dicarbonyls under study in this thesis.

Methylglyoxal

Significance in the Maillard reaction

In recent years, a number of protein crosslinks have been isolated that are thought to involve methylglyoxal (26,30). The physiological concentration of methylglyoxal is thought to range between 256 nM in blood (2.4 μM in diabetics), 1 μM in plasma and 15 μM in urine in healthy human (102-104). However, up to 310 μM has been reported where assay systems have quantified reversibly protein-bound methylglyoxal along with unbound (105). Ninety-nine percent of methylglyoxal is thought to exist in reversibly bound state to protein or other biological ligands (4,105). This leads to the question of whether irreversible adducts through protein-bound amines can form and effect changes in the target protein.

The metabolic flux of methylglyoxal

A major route to methylglyoxal formation is *via* triose phosphates (Figure 1.12) (106-108). This can occur either enzymatically, through triosephosphate isomerase (TIM) (109,110) or methylglyoxal synthase (111), or non-enzymatically, due to the unstable nature of triose phosphates dihydroxyacetone-phosphate (DHAP) and D-glyceraldehyde-3-phosphate (DGAP) (106). Interestingly, neither of the two enzymes mentioned share sequence similarity (112), nor structural identity (113), although they

catalyse almost identical chemistry, only differing in the initial proton that is abstracted (111).

TIM catalyses the inter-conversion of DHAP to DGAP (Figure 1.12). Although highly efficient during the isomerisation process, TIM is not particularly efficient during the elimination reaction to form DGAP. As a result, the phosphoenediolate intermediate can leak from the active site and then be converted to methylglyoxal (Figure 1.12) (109,110).

Methylglyoxal can also be formed enzymatically from glyceraldehyde 3-phosphate (107). Generation of methylglyoxal from glyceraldehyde 3-phosphate has been considered artifactual, but is difficult to negate as initial reports on the subject have not been extended (114). The non-enzymatic formation of methylglyoxal from glyceraldehyde has also been reported (Figure 1.12) (115).

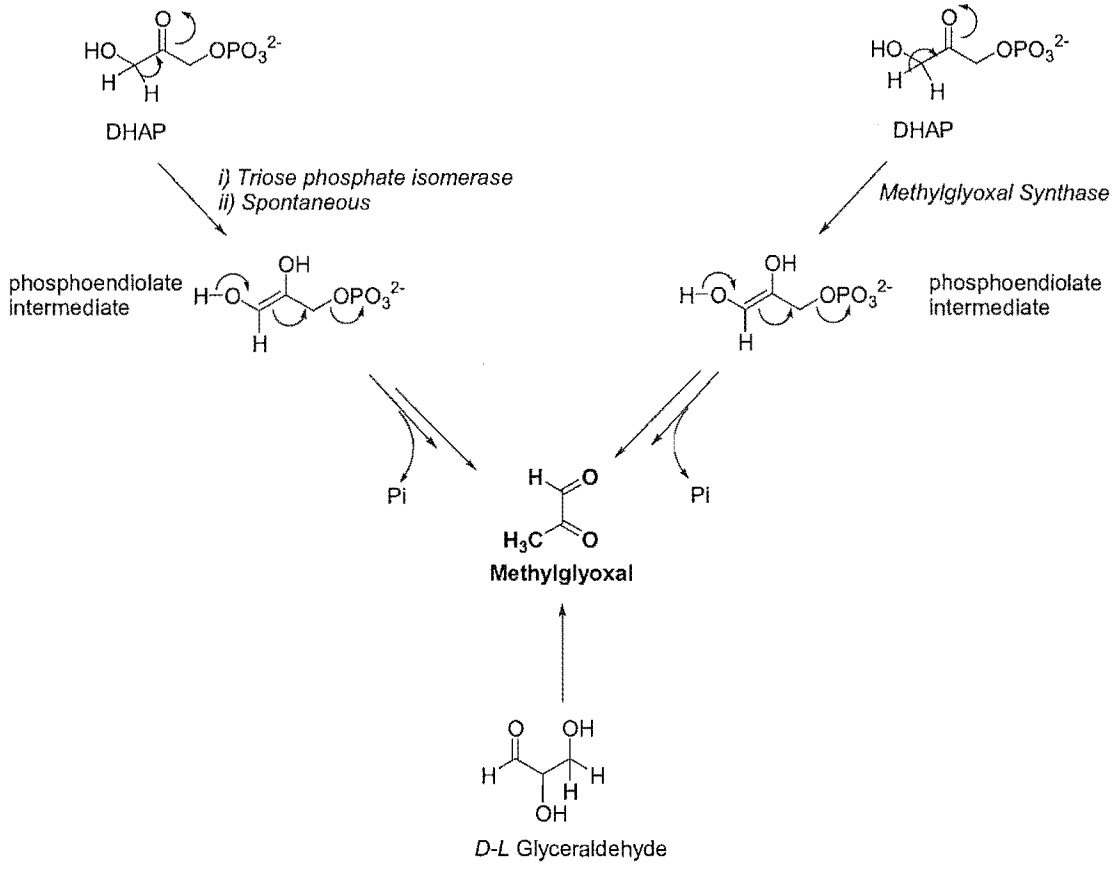


Figure 1.12: The major routes to methylglyoxal formation *in vivo* (110,113,115).

Minor routes to methylglyoxal formation are summarized in Figure 1.13. Two enzymes found to produce methylglyoxal *in vitro*, semicarbazide-sensitive amine oxidase and acetol monooxygenase, have been isolated in rats (Figure 1.13) (116,117). Routes to methylglyoxal formation *via* acetone have also been reported in microbes (118).

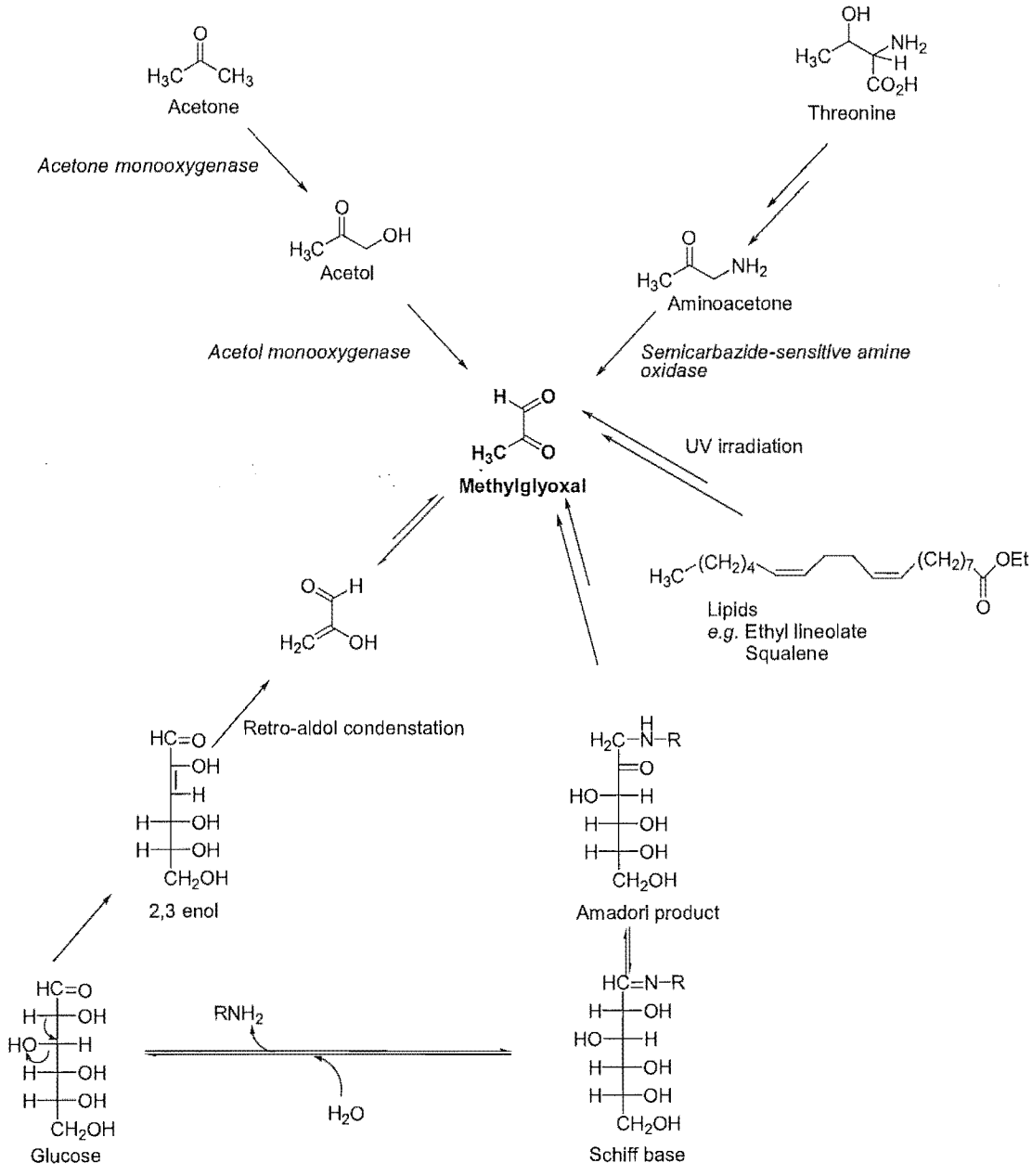


Figure 1.13: Minor routes to formation of methylglyoxal *in vivo* (39,43,116,117,119-121). R, amino acid side chain.

Cells have developed a number of enzymatic (Table 1.1) and non-enzymatic mechanisms to deal with this potentially toxic compound. These enzymes show varying affinity for methylglyoxal (Table 1.1).

Enzyme	Source	Methylglyoxal K_m	Ref
Glyoxalase II	Ubiquitous	66.5 μM	(122)
		130 μM	(123)
Glyoxalase III	*E. coli*	Not reported	(124)
L-Glycol dehydrogenase	*E. aerogenes*	75 mM	(108)
	Hen muscle	Not reported	(125)
Methylglyoxal reductase	*C. beijerinckii*	Not reported	(126)
	C. difficile	Not reported	(127)
	S. cerevisiae	5.88 mM	(108)
	E. coli	4 mM	(127)
Glycerol dehydrogenase	*E. coli*	Not reported	(128)
	K. pneumoniae	Not reported	
Aldose reductase	Human	8 μM	(129)
Aldehyde reductase		1.2 mM	(130)
Aldose reductase like enzyme		130 μM	(131)
Glyoxal oxidase	*P. chyrysosporium*	640 μM	(132)
Formaldehyde dehydrogenase	*C. boibinii*	2.8 mM	(133)
		1.2 mM	(134)
Methylglyoxal dehydrogenase	Sheep liver	40 μM	(135)
	Gram negative bacteria	Not reported	(118)

Table 1.1: Enzymes that have been found to catalyse the break-down of methylglyoxal.

Pathways to the formation and break-down of methylglyoxal have been well documented. A number of pathways can exist within an organism to combat excessive levels of methylglyoxal. It has been difficult to determine which are important, in terms of significant contribution to formation or detoxification of methylglyoxal. Although some groups have studied the flux of methylglyoxal through a metabolic system (136), the significance of particular formation and detoxification pathways of methylglyoxal in all organisms is still unclear. It has been suggested that a barrier to the understanding of metabolic flux, presence, and function of methylglyoxal in biological systems is the poor performance of assay techniques designed to quantify methylglyoxal (107).

Glyoxal

Significance in the Maillard reaction

The physiological concentration of glyoxal has been reported to be 132 μM in healthy human urine and 229 nM in human plasma (104,137). Little is known about whether glyoxal exits as a predominantly free or protein-bound species *in vivo*.

Along with methylglyoxal, glyoxal has become a focus as a potent reagent in Maillard chemistry. This α-dicarbonyl has been shown to be involved in the formation of protein crosslinks (26,30,31). Glyoxal is also responsible for the formation of some non-crosslinking adducts with protein, through lysine and arginine residues (138,139). These latter non-crosslinking AGEs may also effect a change in function, like their crosslinking counterparts.

The metabolic flux of glyoxal

Glyoxal can be formed by many of the routes that have been mentioned for methylglyoxal, such as on autooxidation of glucose, retro-aldol condensation of the Schiff base, metal-catalysed oxidative break-down of Amadori products, and from UV irradiation of lipids (39,42,43,45,56,120,140-143). Glyoxal may also be generated by a free radical mechanism, through reaction of glucose with peroxynitrite (43,144). Interestingly, only one enzyme, a manganese peroxidase enzyme, has been isolated that generates glyoxal (145).

Some enzymes from Table 1.1 also exhibit an affinity for glyoxal, such as glyoxal oxidase, hen *L*-glycol dehydrogenase, and sheep methylglyoxal dehydrogenase (125,135). Substrate studies for two of these enzymes, hen *L*-glycol dehydrogenase, and sheep methylglyoxal dehydrogenase, showed that although glyoxal could bind to these enzymes, the affinity was not as strong as other substrates that were examined (126, 136).

Diacetyl

Significance in the Maillard reaction

Diacetyl has had little coverage in the literature from the perspective of the Maillard reaction. It is of interest to food chemists due to its contribution to the formation of aroma compounds (146). Diacetyl has the capacity to crosslink a model protein, although the structures of these crosslinks have not been elucidated (16). Its presence has been reported in rats, where it is present in concentrations of 85 nM, 48 nM and 389 nM in heart, kidney and liver tissue respectively (147).

The metabolic flux of diacetyl

Diacetyl is formed during the fermentation of milk by *Lactococcus lactis,* where its stability is dependent on a variety of factors, including pH and presence or absence of oxygen (148,149). It can also be formed in beer, *via* the metabolic processes undertaken by the yeast. It has been isolated in model systems that mimic food processing conditions, through fragmentation and subsequent reaction of glucose, and has also been isolated in oxidised edible oil (146,150,151). Production of diacetyl has also been reported during nucleic acid oxidation (152).

Some enzymes that have been mentioned in the detoxification sections of methylglyoxal and glyoxal are also able to turn over diacetyl. These include *L*-glycol dehydrogenase and glycerol dehydrogenase (153,154). Diacetyl reductase has been isolated in microbes and mammals, where it converts diacetyl to acetoin in the presence of NADH (155).

1.7 Inhibition of the Maillard reaction

The Maillard reaction can potentially lead to a number of deleterious effects in the human body, as outlined in sections 1.5 and 1.6. Thus, intervention in the reaction is an important area of research. The implementation of strategies detailed below has shown success in attenuating some physiological manifestations of the Maillard reaction, such as the formation of AGE-crosslinked amyloid plaques (95). However, some that have shown early success have been eliminated as effective intervention approaches due to their chemical instability (156).

As more became known about the differing stages of the Maillard reaction, a number of Maillard reaction inhibitors were designed for intervention at particular points in the Maillard scheme. The points of "attack" are outlined in Figure 1.14. The types of inhibitors will be considered in the classes outlined by Khalifah *et al.* (157).

"Type A" inhibitors compete for amines that are capable of participation in the Maillard reaction. An example is pyridoxal phosphate, which sequesters amines so that they can no longer participate in the Maillard reaction (157). These types of inhibitors are not particularly advantageous from a therapeutic standpoint, due to their non-specific nature with respect to the target amine.

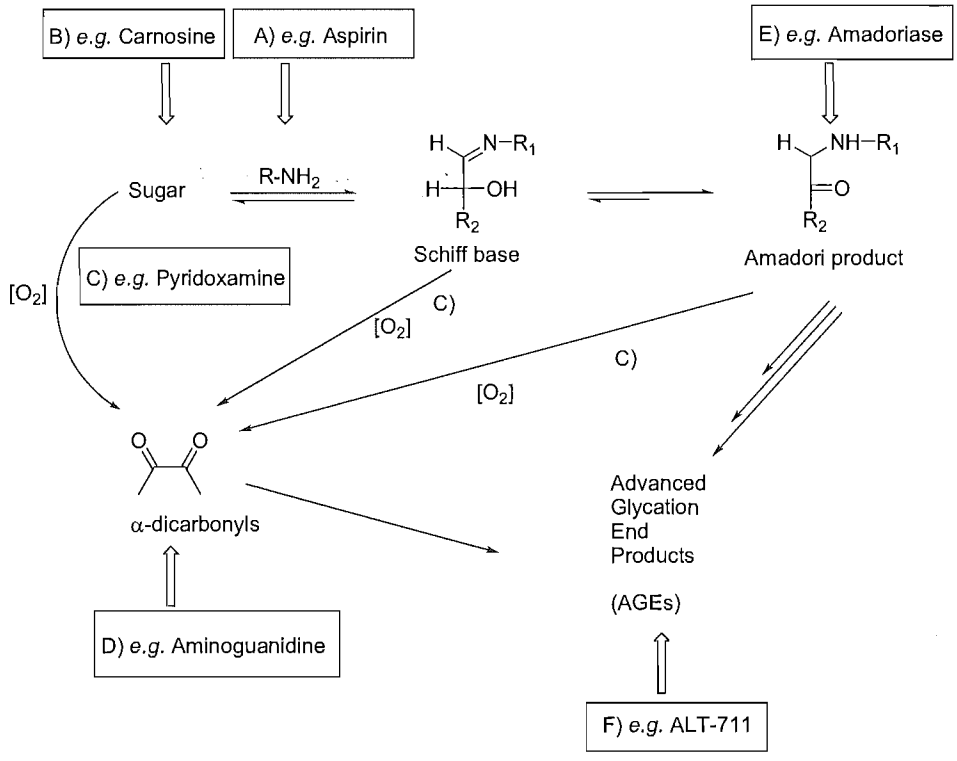


Figure 1.14: The points of intervention in the Maillard scheme. A-F are defined in the text. After Khalifah *et al.* (157). The compounds employed for intervention at various steps are shown in boxes.

"Type B" inhibitors, such as carnosine, work on the same principle, but act to trap the reacting carbonyl molecule, effectively sequestering it from reaction with protein-bound amine (158).

"Type C" inhibitors (Figure 1.14), are a broader class that act to block pathways to formation of other potentially reactive Maillard participants. An example is pyridoxamine that acts to block autooxidation of some carbohydrates through its metal ion chelating ability (159).

"Type D" inhibitors are thought to trap reactive α-dicarbonyl compounds before they can react with the amine (Figure 1.14). One such example is aminoguanidine, which can trap reactive α-dicarbonyls as triazines (120,160).

A class of enzymes have been found to undertake the function of "type E" inhibitors (Figure 1.14). The amadoriase enzymes act on the Amadori product to reverse the early stages of the Maillard reaction (161). Work involving the "type E" inhibitors, the deglycating enzymes that have been shown to act on glycated low molecular weight amines, is still in its infancy. Currently, there is not an enzyme from the class of amadoriases that can act effectively on a glycated protein substrate (161), a shortcoming when considering these enzymes from a therapeutic perspective.

Finally, the "type F" inhibitors are those that have been reported to act by cleaving protein crosslinks, and include phenacylthiazolium bromide and ALT-711 (92,162). The efficacy of these inhibitors has been subject scrutiny with mixed reports on their efficacy (5,93,156,163).

Results from recent studies have challenged our current understanding of the mode of inhibition of some of the above compounds. For example, aminoguanidine was initially thought to react with the Amadori product (164). Later works showed it could react with free sugar to render it unable to react with amine (165), and the dideoxyosone intermediates (166). Until recently, it was thought that the major mode of aminoguanidine inhibition was through binding to α-dicarbonyl compounds acting as a "type D" inhibitor, effectively sequestering them from reaction (160). In the last two years, it has been proposed that aminoguanidine and a number of inhibitors that act at differing stages of the reaction are effective due to their metal-ion chelating ability (*i.e.* a "type C" inhibitor) rather than their proposed action such as dicarbonyl trapping or crosslink breaking (159,167,168).

1.8 Thesis aims

A brief overview of the complex nature of the Maillard reaction has been provided in this introduction. The complexities of the Maillard reaction result in a vast array of products, many of which remain undefined. Protein crosslinks, which comprise a proportion of these products, are thought to be responsible, at least in part, for the progression of some diseases. Surprisingly little is known about the amino acids requirements for this process, and the effect that crosslinking has on protein function. Therefore, this thesis aims to clarify the contribution of protein-bound amines, such as lysine and arginine, to protein crosslinking *via* three α-dicarbonyls, methylglyoxal, glyoxal and diacetyl.

This thesis also probes the activity of a model protein, ribonuclease A (RNase A), following treatment with these dicarbonyls, with a parallel measure on crosslinking. To date, it has been assumed that a change in protein structure, through crosslinking, will result in a change of function of that protein, which forms the basis for the type "F" Maillard inhibitors, the crosslink breakers (section 1.7). Thus, an extensive study was undertaken to test this hypothesis. The proposed α-dicarbonyl trapping compounds aminoguanidine and 3,5-dimethylpyrazole-1-carboxamidine, were included to test their efficacy at inhibiting crosslinking and/or a loss in activity of the RNase A.

The Maillard reaction plays a role in the many aspects of cellular function and therefore, control of this chemistry is essential. The amadoriases have, thus far, not been shown to use glycated protein as a substrate. This may be due to the heavily glycated nature of previously-prepared protein substrate (161). This hypothesis was tested by assessment of the ability of amadoriase I to deglycate RNase A that was mildly glycated with methylglyoxal to create a more physiologically relevant substrate than those trialled in the past. A novel assay approach was developed to determine the efficacy of amadoriase I when using a glycated protein substrate. This assay was then employed to search for enzymes that could deglycate protein from a Pseudomonad, which was isolated from raw milk, a medium rich with sugar and protein.

In addition, two mutants of amadoriase I were created to gain a better understanding of residues in this enzyme that are critical for catalysis. In order to purify both mutant enzymes, along with the wild-type, a reliable time-resolved assay was successfully developed.

1.9 Thesis overview

Chapter two outlines the attempts undertaken to monitor the Maillard reaction, *in vitro*, through assessment of both lysine and α-dicarbonyl concentration over time, and SDS-PAGE electrophoresis.

Chapter three addresses the specificity of the crosslinking reaction by examining the reaction of an arginine containing (lysine-free) and a lysine containing (arginine-free) protein with three α-dicarbonyls, methylglyoxal, glyoxal and diacetyl.

Chapter four examines the effect that glycation and crosslinking by the three α-dicarbonyls have on the catalytic ability of a model protein, RNase A.

Chapter five details the development of a novel assay approach to probing the ability of amadoriase I to use ribonuclease A, that has been mildly glycated with methylglyoxal, as a substrate. Also detailed, is the extension of this method to isolation of a deglycating enzyme from a Pseudomonad grow on casein that had been incubated with lactose. The generation of two amadoriase I mutants and the assay developed for measurement of their activity (along with the wild-type) is also outlined in this chapter.

Chapter six summarises the major findings of this thesis and future directions to be pursued from this work.

Chapter seven outlines all experimental procedures undertaken during this thesis.

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Chapter Two

Monitoring the extent of the Maillard reaction *in vitro*

2.1 Introduction

A number of avenues have been taken by researchers to monitor the progress of the Maillard reaction. This has ranged from measuring the extent of browning, to examining the loss of amino acids that are thought to play a critical role. This chapter aimed to assess the extent of the Maillard reaction in a protein model system through measurement of the concentration of two critical components: α-dicarbonyl compounds and amines, along with the extent of protein crosslinking.

As discussed in chapter one, lysine and arginine residues are thought to play a major role in the Maillard reaction of proteins, due to the fact that they contain amines that are capable of nucleophilic attack on carbonyl compounds. Measurement of lysine concentration, in order to determine the extent of the Maillard reaction, has been undertaken by a number of groups (1-3). Free lysine possesses two NH_2 functionalities within its structure that are amenable to reaction, the α-NH_2 and the ε-NH_2. In a protein, the ε-NH_2 of the lysine residue is available for modification by a carbonyl compound, whereas the α-NH_2 is involved in peptide bonding. Free arginine contains two possible points where a carbonyl can react, the guanidino functionality of arginine and the α-NH_2. In a protein, only the guanidino group remains available. However, as proteins break down, the α-NH_2 terminus may also become available for Maillard chemistry.

Amino acids containing secondary amines such as proline and tryptophan have been extensively studied in the food industry, but less so in the medical arena (4). The secondary amines of proline and of tryptophan have been reported to participate in the Maillard reaction under food processing conditions (4). A decrease in available tryptophan residues in lysozyme has been reported on incubation with glucose or α-dicarbonyl, suggesting that protein-bound tryptophan can participate in the Maillard reaction (5,6). Histidine, which contains a secondary and tertiary amine, has been studied to some extent in the medical area (7,8). A drop in histidine has been reported on mild glycation of β-lactoglobulin by glucose (9).

In this chapter, studies were undertaken to gauge the extent to which proteins are crosslinked during incubation with α-dicarbonyls. In conjunction, the concentration of amine (with a particular focus on lysine) and carbonyl were measured during the reaction process. Comparison of crosslinking rates with amine loss provides an initial insight into relative reaction rates for differing α-dicarbonyls. The methods outlined in this chapter provide the methodology for areas of work undertaken in ensuing chapters and are preceded by a brief review in each area, α-dicarbonyl quantification, lysine quantification and SDS-PAGE.

2.2 Selection of a model protein for *in vitro* studies

The target protein selected to quantify the extent of the Maillard reaction *in vitro* had to meet a number of requirements. It had to be stable, as the study of glycation can involve conditions outside those optimal for protein. It needed to exist in its native state under a variety of potentially unfavorable conditions, such as addition of organic solvent, pH drift and storage at physiological temperatures in an *ex vivo* situation for prolonged periods. Specifically, a protein was required that would be stable at 37°C for up to seven days, and on addition of solvents such as DMSO (Chapter four). Bovine pancreatic ribonuclease A (RNase A, E.C. 3.1.27.5, "A" denotes the predominant form of the enzyme in bovine pancreas) was an excellent candidate as it is highly stable (10), and commercially available. Another advantage of using RNase A is that a great deal is known about this protein, thanks in part to its stability. RNase A was the first protein to have its amino acid sequence correctly determined and was

the third enzyme to have its 3-dimensional structure resolved by X-ray crystallography (10). RNase A, with a molecular weight of 13683 Da, is relatively small and exists as a monomer, although oligomeric forms have been isolated (11-13). The X-ray structure of RNase A is depicted in Figure 2.1. Essentially the protein is comprised of two β-sheets surrounded by three α-helices (12).

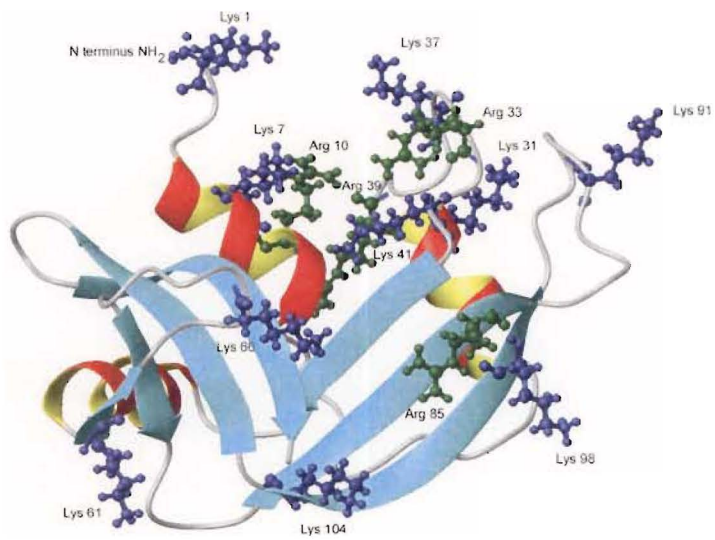


Figure 2.1: The tertiary structure of RNase A at 1.26 Å (14), with lysine residues shown in navy and arginine residues shown in green. The image was produced from the PDB file 7RSA using MOLMOL software (15).

RNase A has been employed by researchers working in the Maillard field for a number of differing purposes such as, studies involving crosslinking (2,16,17), non-crosslinking modifications by sugars (1,18,19), screening of potential Maillard inhibitors (19), and assessment of activity of the native enzyme following glycation (Chapter four) (1,20).

RNase A possesses eleven free amino groups, originating from the ε-amino groups of ten lysine residues, and a terminal α-amino group (Figure 2.1). RNase A also contains four arginine residues within its structure (Figure 2.1). Previous studies have shown modification of some lysine residues (1,16) and arginine residues (21), within RNase A by sugars.

2.3 Attempts to quantify α-dicarbonyl concentration in a model system

2.3.1 Background

This section outlines attempts to quantify α-dicarbonyl concentration following their incubation with RNase A. As α-dicarbonyls are consumed during reaction with functional groups of RNase A (*e.g.* lysine), a decrease in α-dicarbonyl concentration was expected. A search of the literature revealed that the loss of α-dicarbonyl in the context of the Maillard reaction had not been studied. Measurement of α-dicarbonyl concentration in the Maillard area has generally been undertaken from another perspective. Assays have been employed to measure the rate of α-dicarbonyl formation as a result of retro-aldol condensation of glucose, or the Schiff base intermediate formed during the Maillard reaction (22-25).

2.3.2 Literature review of existing methods

In order to quantify α-dicarbonyl concentration, a robust assay was required, and consequently, assays employed to accurately quantify α-dicarbonyl compounds were reviewed. Some have made the observation that a lack of knowledge of the flux of α-dicarbonyls in biological systems is related to inaccurate and unreliable assays (26). Thus, selection of an appropriate assay required careful consideration.

Enzymatic determination of α-dicarbonyl concentration

Some of the very earliest attempts to quantify α-dicarbonyl compounds involved enzyme assays. An assay that was developed in 1957 to measure methylglyoxal has still found use decades later (27,28). This method uses the glyoxalase I enzyme, which catalyses the formation of a thioester, lactoylglutathione, at 240 nm (Figure 2.2) (29). The increase of product is proportional to the amount of α-dicarbonyl present in the sample. Glyoxalase I has been found to have a very broad substrate specificity, exhibiting the ability to bind substituted phenylglyoxal and glyoxal, the former binding more efficiently than methylglyoxal (30,31). This method was not employed however, as the enzyme is specific for aldehydes and ketoaldehydes, and

could not therefore be used to monitor the concentration of the diketo compound diacetyl.

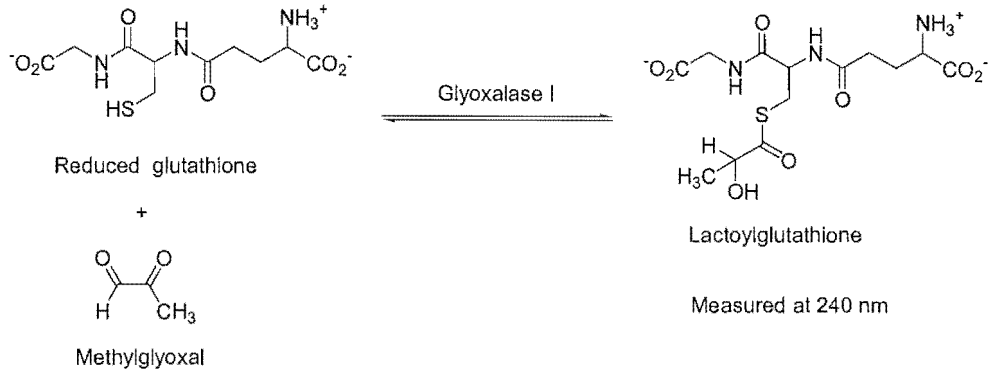


Figure 2.2: The conversion of methylglyoxal and glutathione to lactoylglutathione by glyoxalase I. After Creighton *et al.* (32).

Radioisotopic determination of α-dicarbonyl concentration

Walton and McLean proposed a double-isotope assay to quantify methylglyoxal in tissues. In a solution containing other α-dicarbonyl compounds, the assay was specific for methylglyoxal, providing that the concentration did not exceed 1 mM (33). Glyoxal could not be successful quantified by this approach, and diacetyl was not examined, which excluded this method as a viable option.

Derivitisation agent-based determination of α-dicarbonyl concentration

A number of derivitising agents have been employed in the past to quantify the concentration of α-dicarbonyl compounds (Figure 2.3). The general strategy is to find an agent that will fit the following criteria: highly specific for the target molecule; possesses a unique property such as fluorescence on derivitisation, for effective detection; applicable to a method that is efficient and accessible; and finally, where possible, be commercially available at a reasonable price.

One weakness of derivitisation assays employed to quantify α-dicarbonyls, is the potential for interference from other compounds within the sample (34). For example, a high concentration of trioses have been shown to interfere with 2,4-dinitrophenylhydrazine (2,4 DNP) (Figure 2.3) and aminonicotinic acid assays (35,36). The use of separation techniques such as HPLC reduces the possibility of interference (24,37-44). Additionally, automated HPLC provides a less labour

intensive means for determining α-dicarbonyl concentration. GC can also be employed to separate *o*-phenylenediamine (*o*PDA) derivatives of α-dicarbonyls (45,46). However, some HPLC- and GC-based assays, can be time-consuming due to long derivitisation periods.

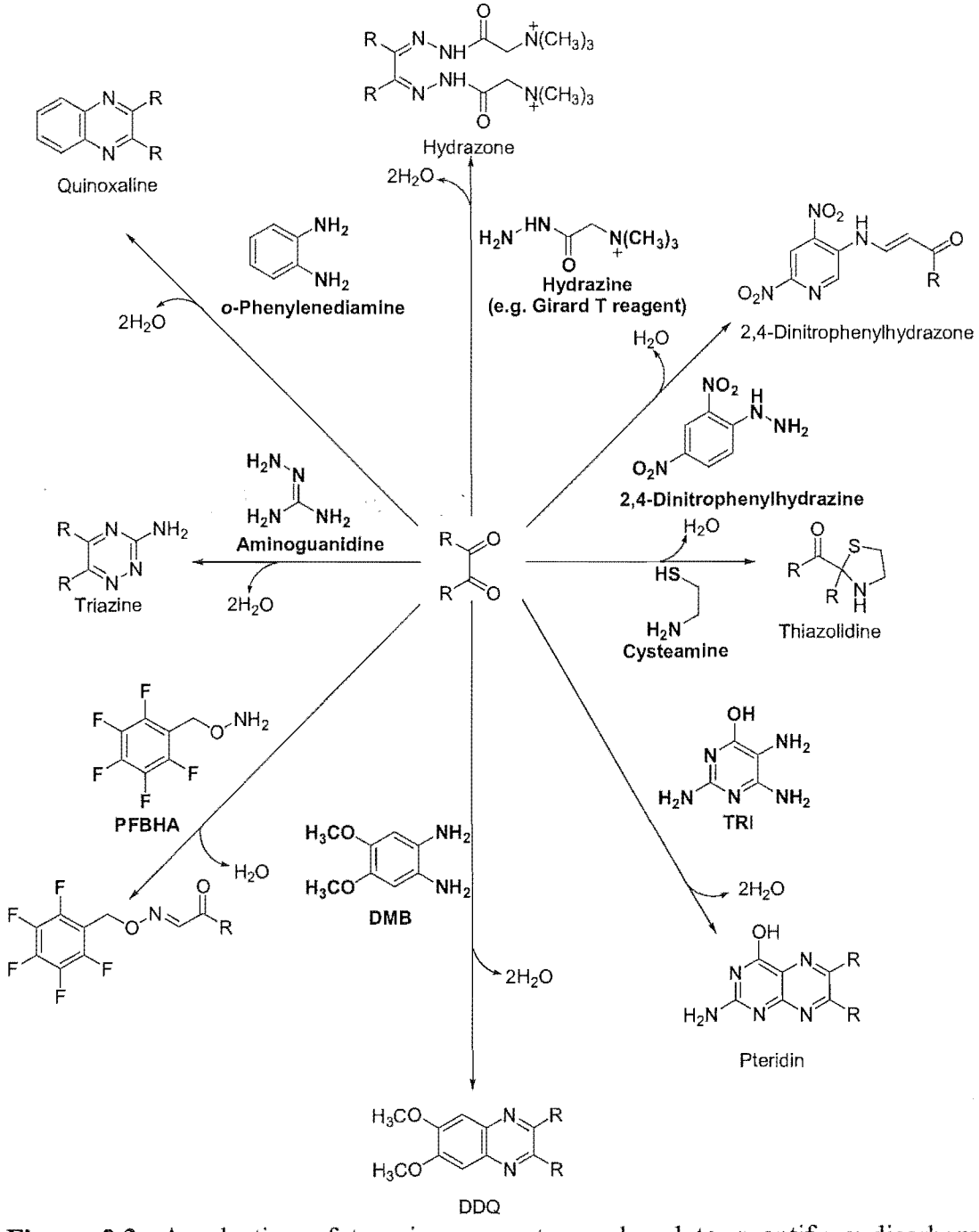


Figure 2.3: A selection of trapping reagents employed to quantify α-dicarbonyl compounds (25,28,34,37,38,47-49). Abbreviations: R, functional group of α-dicarbonyl compound; TRI, 6-hydroxy-2,4,5-triaminopyrimidine; DMB, 1,2-diamino-4,5-dimethoxybenzene; DDQ, 2,3-dimethyl-6,7-dimethoxyquinoxaline; PFBHA, *o*-

(2,3,4,5,6-pentaflurobenzyl)-hydroxylamine. Bold structures and text denote derivitising agents.

In some instances, such as for Girard T and 1,2-diamino-4,5-dimethoxybenzene (DMB) (Figure 2.3), formation of the derivitisation product requires a low pH (23,28,34). Girard T has been employed in the Maillard field (22,50,51). However, lowering the pH may alter the distribution of AGEs in the sample *e.g.* those that are acid labile may survive the assay conditions (section 1.6.1) (3).

The stability of adducts formed after derivitisation is critical for accurate determination of α-dicarbonyl concentration (38). Some adducts, such as triazines that are formed on reaction of α-dicarbonyl with aminoguanidine, (Figure 2.3) have been reported to be unstable under some assay conditions. The reactivity of the trapping reagent must also be considered. Aminoguanidine has been shown to be slower at trapping α-dicarbonyl compounds than other reagents that perform a similar function (25). Both of these issues may lead to erroneous results.

Participation of the derivitising agents themselves in the Maillard reaction has been reported (25). For example, aminoguanidine has been shown to catalyse retro-aldol fragmentation of higher sugars. This can lead to increased amounts of methylglyoxal detected with aminoguanidine over other derivitising agents (25). To achieve accurate measurement of α-dicarbonyl concentration, the time at which the derivitising reagent is added must be carefully considered. Studies have shown that addition of trapping reagent at the start of a reaction can result in interference of the product spectrum by the trapping reagent, due to either its redox capabilities or becasue it may contain amine and thus participate in the Maillard reaction (25). Alternatively, addition of derivitising agent at various time points of sampling can affect the products (25,40).

2.3.3 o*-Phenylenediamine assay (oPDA) of glycated samples*

Why oPDA?

The derivitising agent *o*PDA (Figure 2.3) was selected to measure the α-dicarbonyl concentration in α-dicarbonyl-treated lysine or RNase A incubations. The method employed was derived from Li and Kenyon, and involved quantification of the

resultant quinoxaline derivatives by spectrophotometric assay (52). The conditions of the assay were favourable for quantification of reversibly bound methylglyoxal (38). This method was selected on the basis of its sensitivity, in comparison to existing methods (*e.g.* 2,4 DNP), and that it avoids extraction that is required for some GC and HPLC methods (52). Other factors that contributed to its selection were that the adducts formed are found to be stable (25), that it was fast and required only readily available equipment. Finally, *o*PDA was commercially available at a reasonable price.

Quantification of α-dicarbonyl concentration in lysine + α-dicarbonyl model incubations

The *o*PDA method was first investigated with lysine, which contains two free amine moieties, in order to investigate the efficacy of this method at gauging the extent of the Maillard reaction, before progressing onto a more complex model system involving RNase A.

Following inspection of the calibration curves for each α-dicarbonyl (Figure 2.4 A, B, C), final assay concentrations were selected for the assay that were in the linear region of the calibration curves. Lysine samples containing α-dicarbonyl, following incubation, were diluted appropriately and the concentration of α-dicarbonyl determined. The assay included a no-lysine control incubation, which gauged the quantity of α-dicarbonyl loss through non-Maillard mechanisms *e.g.* self-condensation of the α-dicarbonyls, or their reaction with water. A no-α-dicarbonyl incubation was included to eliminate any possible interference in the assay by lysine itself. Interference by reaction products generated during incubation of lysine with α-dicarbonyl, and also the α-dicarbonyl itself, at the wavelength the assay was performed and was found to be negligible.

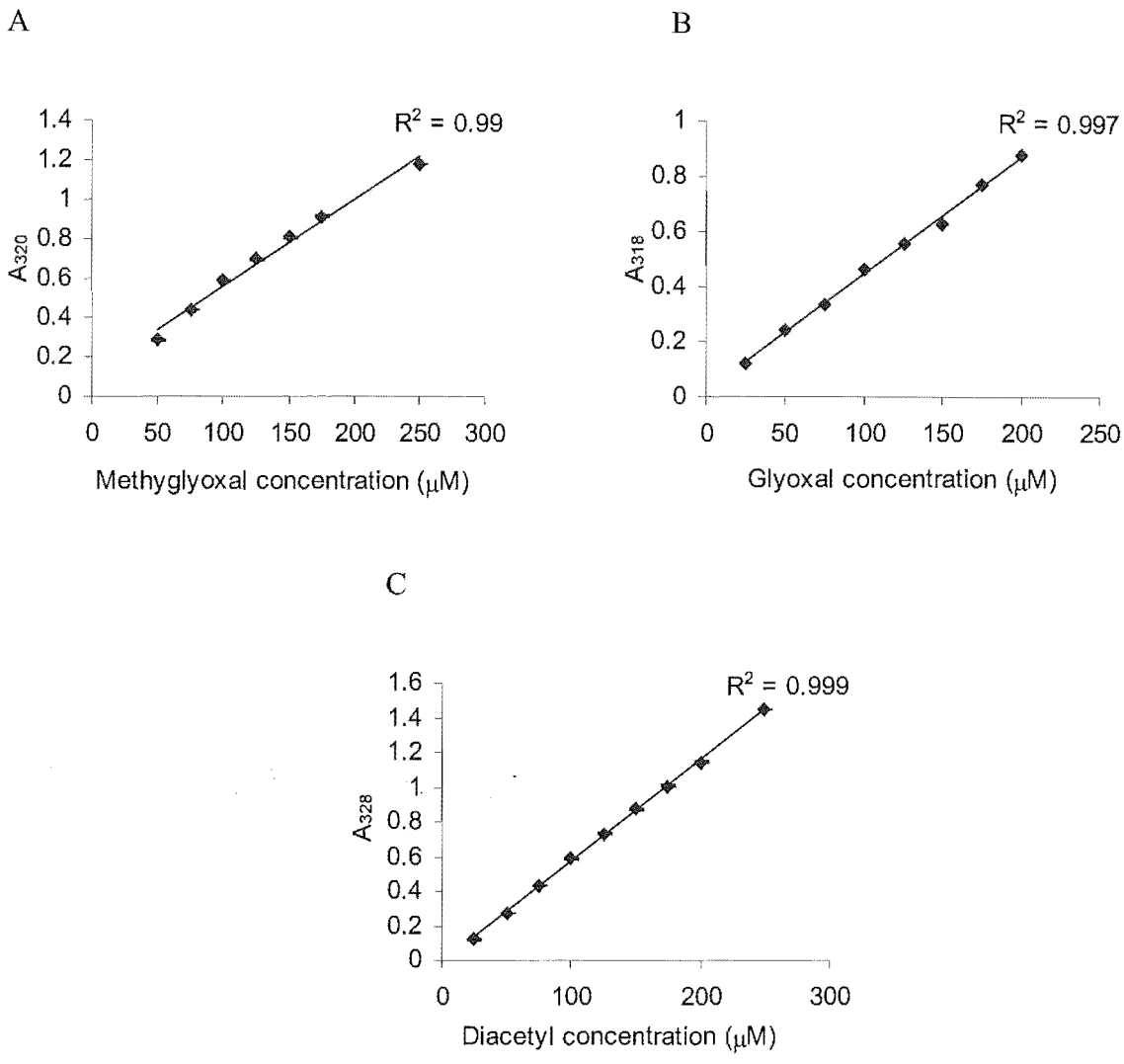


Figure 2.4: Calibration curves for A, methylglyoxal; B, glyoxal; C, diacetyl; with *o*PDA, constructed to determine the concentration of each respective dicarbonyl after incubation with lysine or protein. All points are an average of triplicate samples with error expressed as standard error of the mean. (In the above case error is small and thus error bars are obscured by each data point).

Figure 2.5 A shows that methylglyoxal exhibits a noticeable decrease in concentration over time, with only 30% of methylglyoxal remaining after 7 days of incubation with lysine, suggesting that approximately 70% of methylglyoxal is consumed. However, the methylglyoxal-only samples also exhibited a drop in concentration, indicating a consumption of approximately 30% of methylglyoxal by non-Maillard reactions, such as polymerisation reactions (53). This is not unexpected, as all dicarbonyls studied here have been shown to polymerise (53-56).

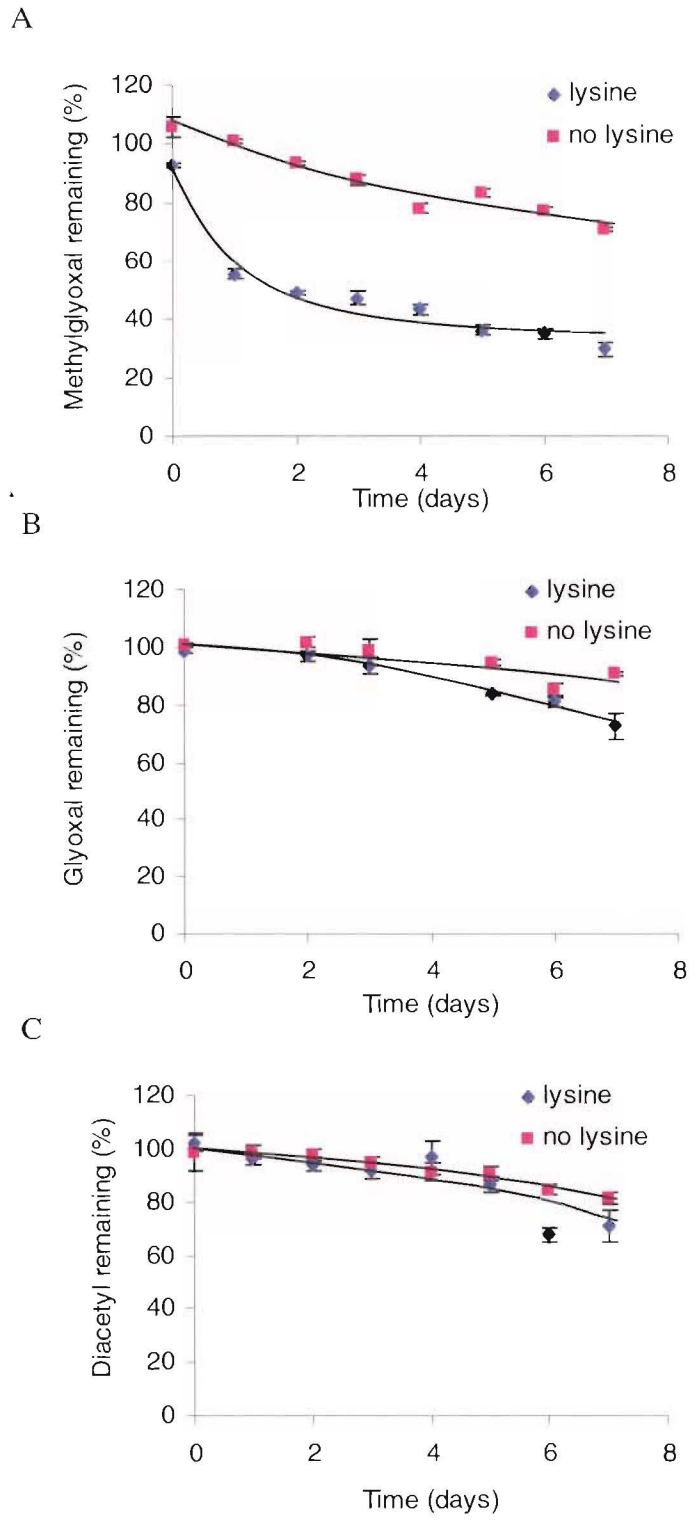


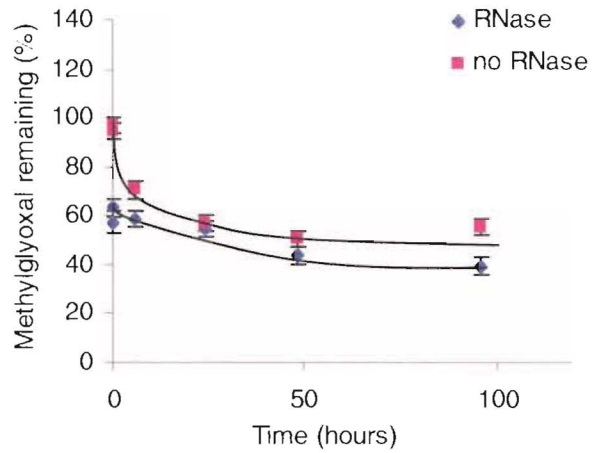
Figure 2.5: Percentage α-dicarbonyl remaining over time for A, methylglyoxal; B, glyoxal; C, diacetyl; with *o*PDA, in the presence or absence of lysine. Readings for each time point represent an average of triplicate samples. Error is expressed as standard error of the mean for triplicate samples.

Glyoxal and diacetyl also showed a decrease with and without lysine (Figure 2.5 B and C). The difference in α-dicarbonyl concentration between samples containing amine and those not, is much smaller. After 7 days of incubation, glyoxal concentration was at approximately 70% with the no-lysine control at around 90%. This was mirrored by diacetyl, with the only difference being a larger decrease in the no-lysine samples (85%). Hence, the decrease in glyoxal and diacetyl concentration, under the incubation conditions employed in this work, was *via* non-Maillard processes. From this work, the order of α-dicarbonyl reactivity with amine appears to be methylglyoxal >> diacetyl ≈ glyoxal.

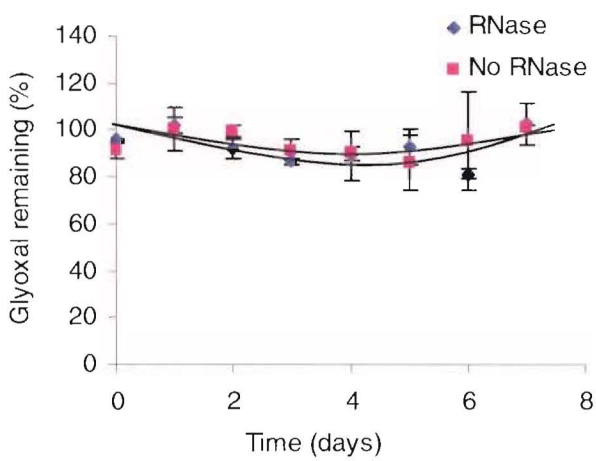
Quantification of α-dicarbonyl concentration of RNase A + α-dicarbonyl incubations

The next phase involved examining α-dicarbonyl concentration in a more complex, physiologically relevant system involving protein. RNase A (25 mg/mL, 20 mM amine) was incubated with α-dicarbonyl (130 mM, 1 mol amine: 6 mol α-dicarbonyl). This high ratio was selected to ensure glycation occurred over an appropriate period of time with minimal decrease in protein concentration due to break-down under the incubation conditions (16). On inspection of Figure 2.6, as with the lysine incubations, it becomes clear from the methylglyoxal and diacetyl assays (Figure 2.6 A and C respectively) that α-dicarbonyl loss in the absence of protein is similar to that in incubations that contain protein. Glyoxal concentration does not appear to decrease over time in the presence or absence of protein (Figure 2.6 B). Methylglyoxal was again the most reactive, with around 40% percent of methylglyoxal consumed immediately on incubation with protein, whereas the control was effectively 100%. Diacetyl appeared to be less reactive compared to methylglyoxal, reaching around 45% consumption after 1 day of incubation under the same conditions.

A



B



C

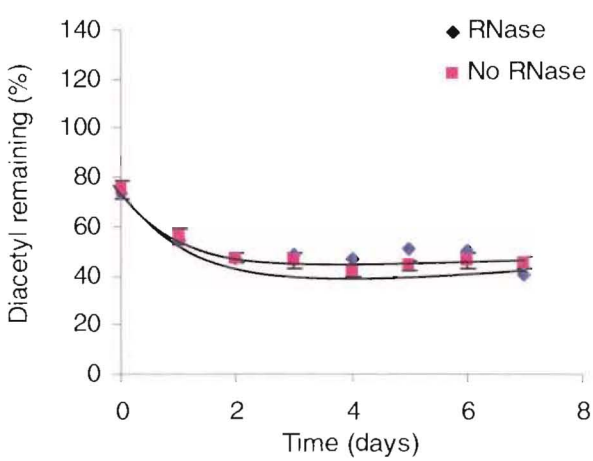


Figure 2.6: Percentage α-dicarbonyl remaining over time for A, methylglyoxal; B, glyoxal; C, diacetyl; with *o*PDA in the presence or absence of RNase A. Readings for each time point represent an average of three samples. Error is expressed as standard error of the mean of triplicate samples.

2.3.4 p-Hydroxybenzoic acid hydrazide (HBH) assay of glycated samples

In order to corroborate the results gained for the *o*PDA method, a second method was trialled to determine α-dicarbonyl concentration. This method involved derivitisation of the α-dicarbonyl in question with HBH (Figure 2.7), to yield a coloured product (57,58). It is not reported whether this measures either free or both free and reversibly bound α-dicarbonyl. This method provided a simple, relatively fast means for measuring a wide concentration of α-dicarbonyls and did not require any specialised equipment to run.

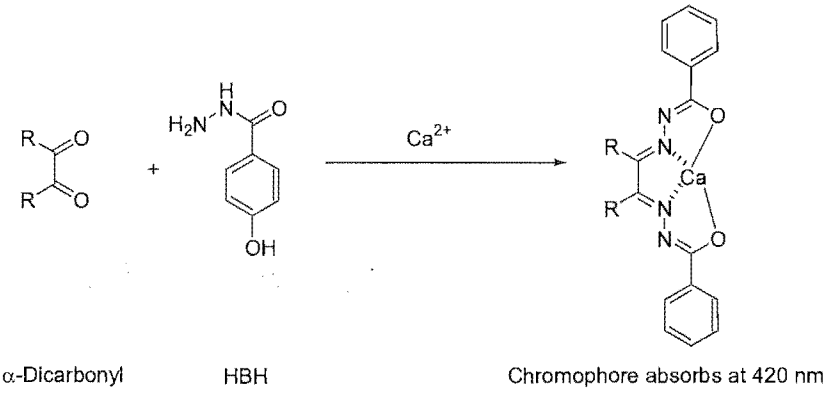


Figure 2.7: The reaction between *p*-hydroxybenzoic acid hydrazide (HBH) and an α-dicarbonyl (57,58).

The assay was adapted to measure the concentration of the three α-dicarbonyls of interest. The source paper outlined two methods for concentration determination, only differing in concentration range for the calibration curves (57). Calibration curves for both procedures are outlined in Appendix one, Figure 1.

The first form of the literature assay required high concentrations of α-dicarbonyl. Due to the fact that incubations were prepared in small amounts, low volumes of each initial incubation were used, in order to conserve sample. As dilution of the sample for assay was minimal, only small volumes were used throughout most of the procedure. As a result, this method introduced a large amount of error (Appendix one, Figure 2). Therefore other than an immediate drop in concentration of α-dicarbonyls, little can be drawn from these results.

The second method used a higher concentration of HBH and lower concentration of α-dicarbonyl, so that the initial incubation mixture could be diluted into a larger volume of solution, in an attempt to circumvent the error issue. A representative graph of these analyses is outlined in Figure 2.8. These experiments, in general, resulted in less error within the assay than the first HBH method (see Appendix one, Figure 3 for calibration curves). The results also corroborated those obtained using *o*PDA as a derivitising agent. The α-dicarbonyl-only incubations paralleled those α-dicarbonyl incubations that also contained amine in the form of protein (Figure 2.8). The results with methylglyoxal showed similar results to those depicted with diacetyl, a concomitant decrease in the methylglyoxal only and methylglyoxal + RNase A samples. The results for glyoxal are reported in Appendix one, Figure 4. This suggests that polymerisation of α-dicarbonyls, in the absence of the Maillard reaction, results in an almost equivalent loss of reactant. Thus, the loss of α-dicarbonyl on addition of protein does not provide a useful measure of the Maillard reaction rate.

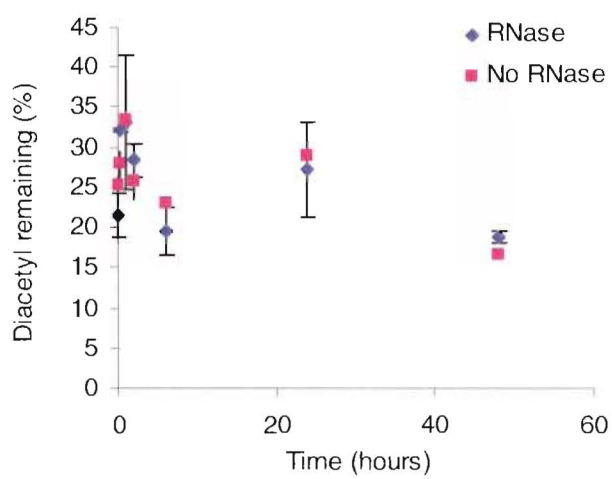


Figure 2.8: Percentage diacetyl remaining over time, in presence or absence of RNase A in incubations. Readings for each time point represents an average of three samples. Error is expressed as standard error of the mean of triplicate samples.

This section has detailed attempts to quantify the α-dicarbonyl concentration during the Maillard reaction. Initial studies with *o*PDA highlighted the reactive nature of these molecules, where in some cases the α-dicarbonyls were equally as efficient at reacting with water and/or themselves as with free lysine or protein. This finding was corroborated by experiments using an alternative derivitising agent, HBH. Thus, this

approach to quantifying the extent of the Maillard reaction appears to be limited by the reactive nature of these α-dicarbonyls in solution.

2.4 Attempts to quantify lysine concentration in a model system

2.4.1 Why lysine?

Since quantification of α-dicarbonyls met with limited success, the other critical reaction substituent, the lysine residue, was quantified *via* its amine moiety. Lysine was selected over arginine because of difficulties that can be encountered when measuring arginine concentration (59).

2.4.2 Review of existing methods of lysine quantification

Monitoring the decrease of lysine residues over time in protein that has been reacted with a carbonyl compound is one way to gauge the rate of the reaction. Using such an approach, the amino terminus has also been shown to participate in the Maillard reaction (1). In some proteins, initial Schiff base formation has been reported to be more favourable with the *N*-terminus than with protein bound lysine. However, the subsequent formation of Amadori product at the *N*-terminus occurs to a lesser extent (1). There are many approaches that have been taken to quantify lysine content. The techniques examined below are those that have been applied in the Maillard arena.

Biological and microbial approaches to lysine quantification

As lysine is an essential amino acid, many researchers have been interested in the conditions where this amino acid is limiting to the growth of an organism. There is a raft of biological assays that have been undertaken to assess how effective a food source can be at providing lysine and thus stimulating growth (60,61). These assays are specific to lysine residues, differentiating between it and the *N*-terminus. Often these assays involve a number of variables and can be time-consuming and expensive (62).

Microbiological approaches to determining lysine concentration have been suggested which would involve measurement of activity of lysine-induced enzymes within bacteria. However, this can cause problems with enzymes that can act on glycated lysine and thus may be unsuitable for estimation of lysine where the Maillard reaction is involved (60). Particular care must also be undertaken to exclude any contaminating bacteria which may interfere with the assay.

Another approach to quantifying lysine has involved lysine oxidase, which catalyses the conversion of L-lysine, water and oxygen to 2-oxo-6-aminocaproic acid, hydrogen peroxide and ammonia (63). The reaction products have been measured a number of ways including spectrophotometry, chemiluminesence, fluorimetry and electrochemistry (63).

Maillard product dependent approaches to lysine quantification

A traditional method for estimation of lysine content, amino acid analysis, involves refluxing the glycated protein in 6 M acid at 110°C (18,64). For reasons outlined in the Chapter one, this may lead to inaccuracies in measured lysine content. However, furosine, a product formed under amino acid digest conditions, has also been used as a marker to quantify the extent of the Maillard reaction and is easily separated and quantified (Figure 2.9) (60). If quantitative conversion of Maillard reaction products to furosine is assumed, quantification of reactive lysine (lysine with the ε-NH_2 available) and inactivated lysine can be achieved (either as an early or late Maillard reaction product) (65). This approach has been used to measure lysine damage in whole milk and whey proteins (65,66). However, it can be difficult to determine a decrease in lysine *via* this method as it assumes that all lysine residues are present as fructoselysine, precluding measurement of other Maillard reaction products (67).

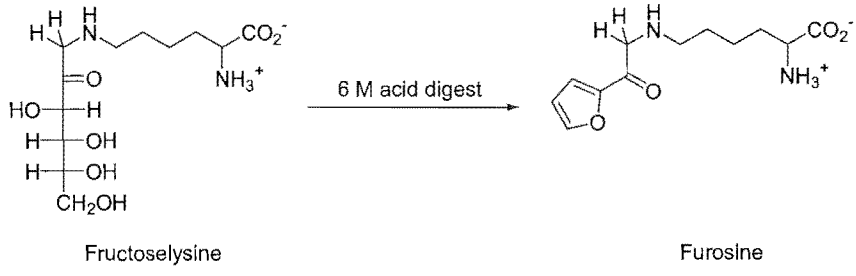


Fig 2.9: Formation of furosine on reaction with fructoselysine (an Amadori product) (60).

Dye-based approaches to lysine quantification

Dye binding assays have received limited coverage in the literature in terms of quantifying lysine. One example is Acid Orange 12 (Figure 2.10), an azo dye that contains a negative charge which is though to interact with the positively charged amine of lysine (along with those on arginine and histidine) (60). One study favoured this assay over other assay methods due to its convenience, speed and higher sensitivity relative to the existing methods available at the time (68). This method does, however, require the use of a low pH (pH, 1.25), which may alter the proportions of the Maillard reaction products of lysine (69).

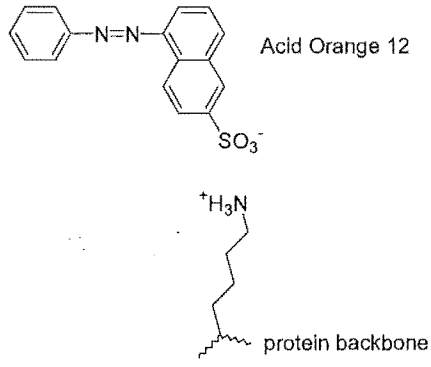


Figure 2.10: The structure of Acid Orange 12 which allows for possible interaction with lysine (60).

Derivitisation-based methods to quantify lysine

All of the assays discussed thus far have involved quantification of different forms of lysine. A number of different groupings of lysine can be considered: "reactive lysine" which contains a free ε-NH_2 that will react under assay conditions; "available lysine", which is biologically available for protein synthesis; and "total lysine", which comprises reacted and unreacted lysine (60). The assays outlined below measure "reactive lysine", following incubation of lysine or protein with carbohydrates. Many of these assays rely on the principle of derivitising lysine in order to obtain a compound that can be determined by spectrophotometric or chromatographic means (70-72). Some of these derivitisation methods, however, involve addition of acid or base which may result in the disruption of protein structure and a change in the profile of the Maillard products in the solution studied (62).

1-Fluoro-2,4-dinitrobenzene (FDNB) was the first derivitising agent employed for measuring reactive lysine (Figure 2.11) (69). There are difficulties associated with this method, such as considerable inter-lab variation of values generated by this method (65,69).

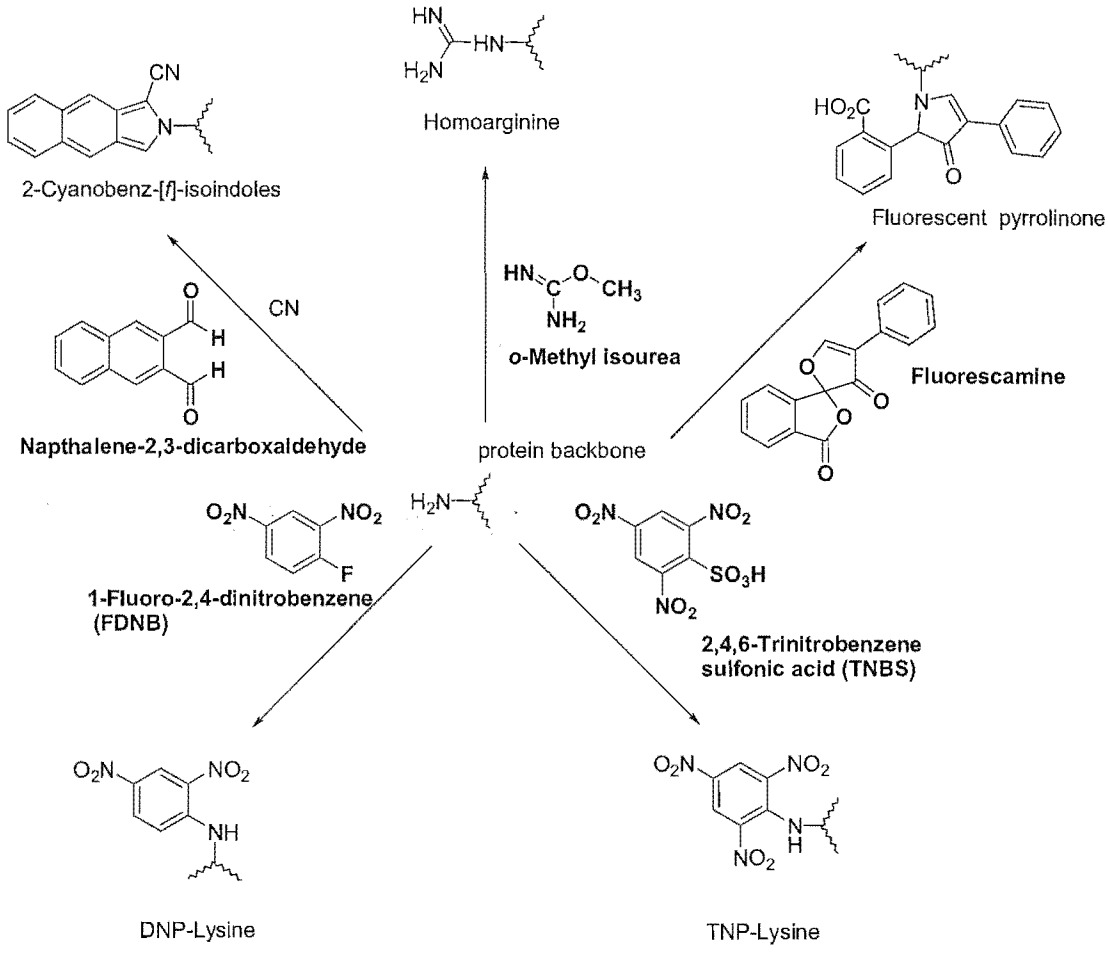


Figure 2.11: Common derivitising agents employed in the literature to quantify "reactive lysine" (60,71,76,79). Derivitising reagents are depicted in bold. Abbreviations: DNP, dinitrophenyl; TNP, trinitrophenyl.

Another derivitising agent used extensively in the literature to quantify lysine is 2,4,6-trinitrobenzene sulfonic acid (TNBS, Figure 2.11) (71). It has also been used to modify lysine in studies probing the mechanism of some enzymes (73-75). *o*-Methyl isourea, also used to quantify lysine, converts lysine to the acid-stable homoarginine (Figure 2.11). Some have suggested, however, that this method can lead to underestimation due to incomplete guanidination of lysine (72). Fluoresecamine assays (Figure 2.11) have the advantage of being fast and sensitive, relative to older

methods involving the use of ninhydrin (76,77). However, these assays can encounter problems due to differing interference intensities of derivitised amino acids (78). Naphthalene-2,3-dicarboxaldehyde (Figure 2.11) has also been used to quantify lysine (78). It reacts with primary amines (in the presence of cyanide) to yield a stable fluorescent 2-substituted cyanobenz- (*f*)-isoindoles. This assay is even more sensitive than fluorescamine and does not require a long derivitisation period (78).

o-Phthaldialdehyde (*o*PA) was first used to derivitise amino acids for a fluorimetric assay (Figure 2.12) (80). The 1-alkylthio-2-alkyl-substitued isoindoles are the products responsible for fluorescence observed on reaction of β-mercaptoethanol with *o*PA, and is the product measured in spectrophotometric assays (Figure 2.12) (81-84). Fluorescence is reported to be highly dependent on the presence of β-mercaptoethanol, as is the spectrophotometric variant of the assay (82). The spectrophotometric assay using *o*PA provides a simple and relatively fast method of lysine quantification. Another factor in the favour of this assay is that there is no requirement for addition of acid (1,19,64,85,86). Due to these considerations, this assay was selected to quantify lysine in studies outlined below.

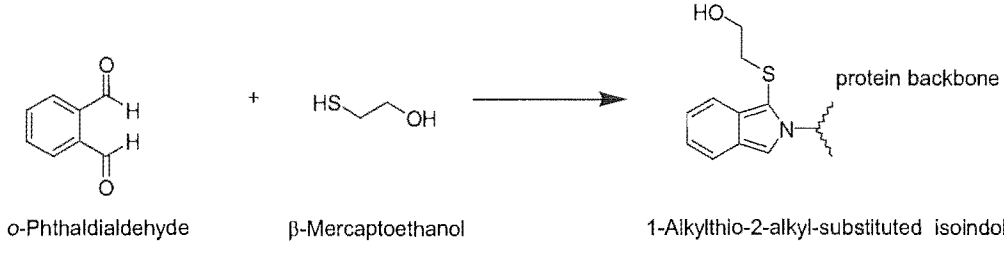


Figure 2.12: Reaction of *o*PA with amine and β-mercaptoethanol. After Simons and Johnson (81) and Švedas *et al.* (82).

2.4.3 Determination of lysine content of RNase A + α-dicarbonyl incubations

Background to the o-phthaldialdehyde (oPA) assay

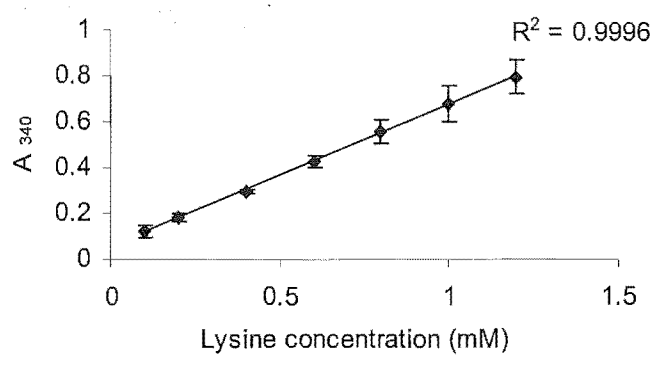
Since its inception, the *o*PA method has been extensively used in HPLC-based assays in order to quantify amine concentration (3,87-89). In the case of RNase A, nine of the eleven available amines react with *o*PA using a spectrophotometric derivative of the assay (16,17). It has been proposed that the remaining two are unable to react due to steric considerations (90).

Incubation conditions and calibration curve construction to determine lysine concentration

The *o*PA method used for lysine estimation of glycated RNase A follows the method developed by Fayle *et al.*, building on an existing method (16,84).

An RNase A concentration of 25 mg/mL (20 mM amine equivalents, lysine and *N*-termius), a concentration previously used in this laboratory (16,91), was used for the experiments detailed below. A starting concentration of 130 mM was selected for each α-dicarbonyl compound. The calibration curves were constructed in order to calculate the concentration of lysine residues in glycated samples (Figure 2.13). Interpolation of lysine concentration for the absorbance of 2 mg/mL RNase A from a lysine calibration curve (Figure 2.13 B) confirms that nine of the eleven available amine groups present in RNase A react with *o*PA.

A



B

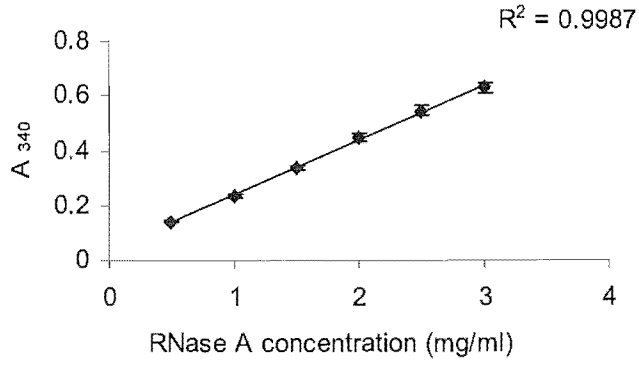
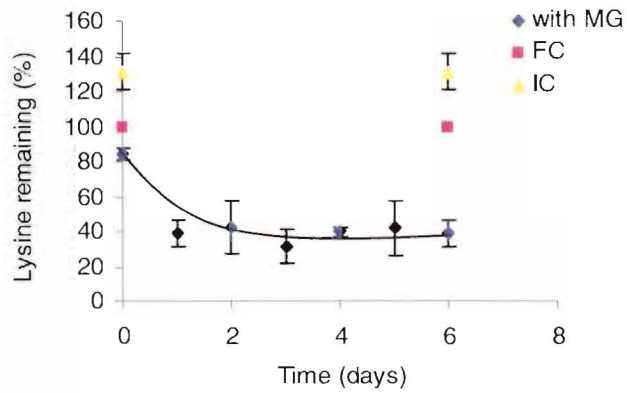


Figure 2.13: Calibration curves for lysine estimation *via* the *o*PA assay for A, RNase A; B, lysine. Readings are an average of triplicate experiments. Within each experiment, triplicate samples of each concentration were prepared. Error is expressed as the standard error of the mean of triplicate experiments.

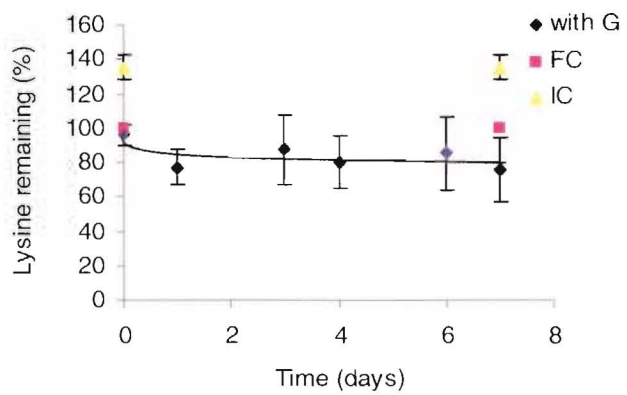
Lysine concentration in RNase A + α-dicarbonyl incubations

The decrease in lysine concentration in protein incubated with methylglyoxal, glyoxal or diacetyl was measured using the *o*PA method (Figure 2.14).

A



B



C

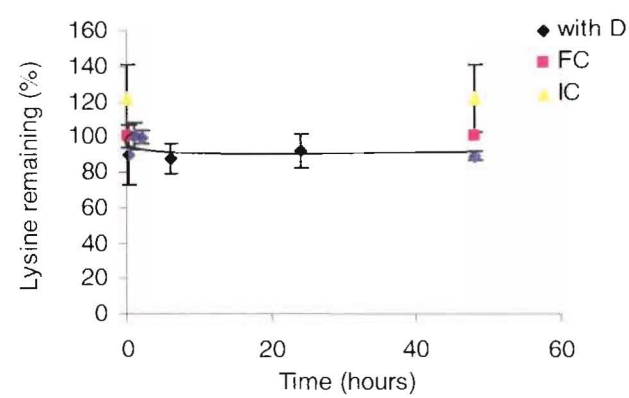


Figure 2.14: Percentage lysine remaining in RNase A on incubation with A methylglyoxal; B, glyoxal; C, diacetyl. Abbreviations: MG, methylglyoxal; G, glyoxal; D, diacetyl; FC, frozen control; IC, incubated control. Each point is an average of triplicate experiments with triplicate readings of time points within each assay. Readings are expressed as percentage relative to frozen control for each α-dicarbonyl. Error is expressed as standard error of the mean for triplicate experiments.

The majority of amine modification of RNase A occurred within the first day of glycation with methylglyoxal (Figure 2.14 A). Decreases in amine concentration after incubation with glyoxal and diacetyl are less obvious. Under the experimental conditions employed in this work, little change in free amine is seen over time (with respect to each day and the frozen control) as shown in Figure 2.14 B and C. However, on closer inspection, there is a large difference in the final day incubation containing α-dicarbonyl, compared those incubations that did not contain α-dicarbonyl (*i.e.* the incubated control).

In almost all assays, it was noted that the incubated control had a higher concentration of amine than the frozen control (Figure 2.14 A, B, C). The only experimental difference between the two controls was that one had been incubated at 37°C for several days, whilst the other was stored immediately at -20°C. Figure 2.15 emphasises this point, showing that lysine is lost to some extent on treatment of RNase A with each α-dicarbonyl.

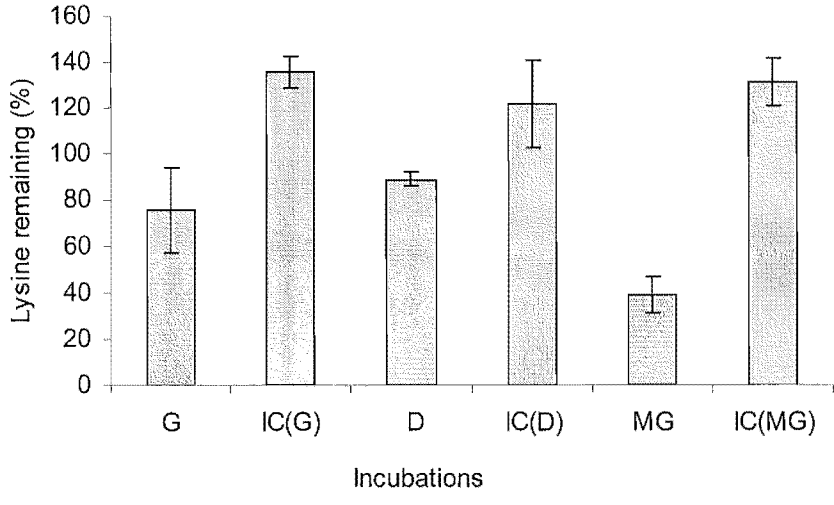


Figure 2.15: Percentage of free amine groups remaining in RNase A incubated in the presence (G, D, MG) or absence of α-dicarbonyl (IC(G), IC(D), ICMG)). Abbreviations: G, glyoxal; IC(G), incubated control from glyoxal experiments; D, diacetyl, IC(D) incubated control from diacetyl experiments; MG, methylglyoxal, IC(MG) incubated control in methylglyoxal experiments. Readings are expressed as percentage relative to frozen control for each dicarbonyl. Error is expressed as standard error of the mean for triplicate experiments.

An explanation for the apparent elevated levels of lysine residues detected in the incubated control may be due to the break-down of RNase A. Break-down of RNase A may generate available amine termini that were previously involved in peptide bonds. These termini will be detected in the assay as *o*PA can react with free α-NH_2 leading to inflated lysine values.

The competing processes of protein break-down and amine reaction with α-dicarbonyl compounds may result in an underestimation of amine loss, and thus misleading conclusions (Figure 2.16).

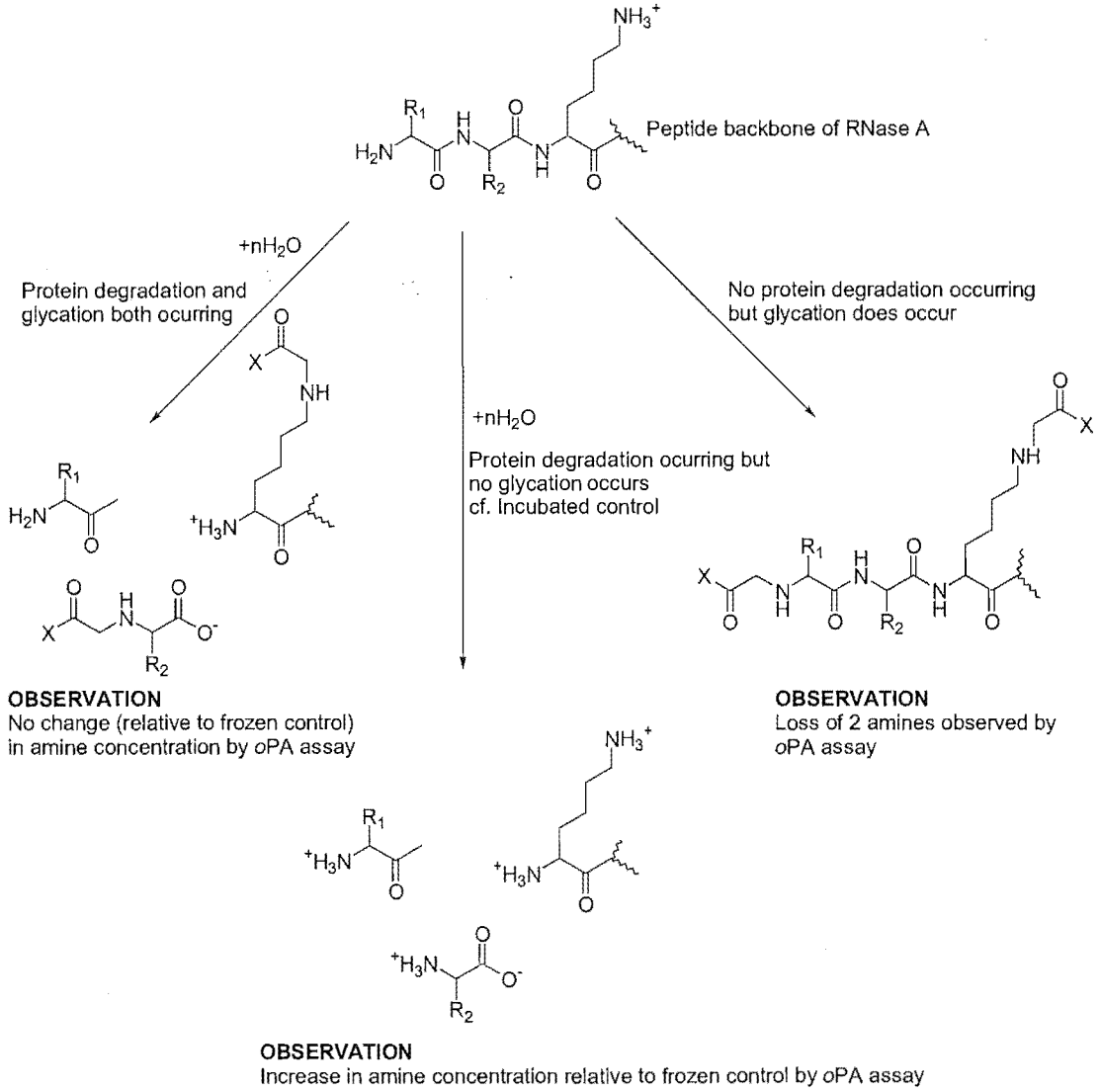


Figure 2.16: The relationship of RNase A break-down to observations made during amine count experiments. R_1, R_2 = amino acid side chain, X = remainder of carbonyl moiety.

Interestingly, RNase A, a protein known for its stability, appears to be susceptible to break-down. Thus, this effect may be more pronounced in less stable proteins and the results from *in vitro* experiments must be interpreted with caution. At least one report has shown protein break-down during *in vitro* Maillard studies, when examining wheat proteins (92).

On inspection of the results from this section and the previous section involving α-dicarbonyl quantification, a consistent picture emerges. Although both methods showed some limitations, each showed that of the three α-dicarbonyls, methylglyoxal was the most reactive, and that glyoxal and diacetyl reactivites were evenly matched in the context of the Maillard reaction.

2.5 Crosslinking of RNase A as assessed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

In order to corroborate the α-dicarbonyl and lysine studies undertaken to gauge the extent of the Maillard reaction, SDS-PAGE was employed to assess the macromolecular effect of reaction of α-dicarbonyls with protein. SDS-PAGE enables the detection of high molecular weight aggregates that have been reported during the Maillard reaction (16). SDS-PAGE allows for the resolution of multimeric states of a protein under reducing conditions which are unfavourable for disulfide bonds, thus, only non-disulfide crosslinks are detected. In tandem with lysine and α-dicarbonyl estimations, this methodology provides a powerful tool for further probing the extent of the Maillard reaction and provide clues to the reactivities of the α-dicarbonyl compounds.

2.5.1 Background to SDS-PAGE methodology

Electrophoresis is a relatively simple, yet highly sensitive, separation technique (93). For SDS-PAGE, the percentage of acrylamide and bis-acrylamide dictates the pore size of the gel constructed using these compounds. Gradient gels differ in acrylamide concentration within the gel itself (such as 8-16% gels) results in large pores at the top of the gel, where acrylamide concentration is low and small pores at the bottom of the

gel where acrylamide concentration is much higher. The advantage of varying the concentration of acrylamide allows for the separation of proteins from a wide range of molecular weight, on a relatively small gel, in a short period of time. The addition of SDS increases the solubility of the sample and confers a uniform negative charge density on the protein allowing for migration solely based on size, not charge (93). SDS-PAGE crosslinking has received some coverage in the medical arena with respect to the Maillard reaction (2,7,94), and has also begun to find use in food chemistry studies (92).

2.5.2 SDS-PAGE analysis of RNase A incubated with α-dicarbonyl

SDS-PAGE was used to assess the macromolecular structure of RNase A following glycation. Figure 2.17 shows a typical result depicting the extent to which RNase A was crosslinked by methylglyoxal, glyoxal and diacetyl.

Some of these results appear to confirm the theory that was proposed on inspection of the lysine count data, that the RNase A was breaking down over time. This is manifested on the SDS-PAGE gels by a large degree of smearing under the molecular weight of RNase A, indicating protein fragments. Figure 2.17 C highlights this particularly clearly, with a steady increase in fragments over time (beginning from 2 hours of incubation).

On inspection of Figure 2.17, it is evident that a small proportion of control RNase A is present in the dimeric form. This dimeric form of RNase A is well documented in the literature and is thought to be formed during the purification of RNase A (11,95,96). Recent work has found that RNase A exists in its domain swapped (dimeric) form at a near neutral pH (97).

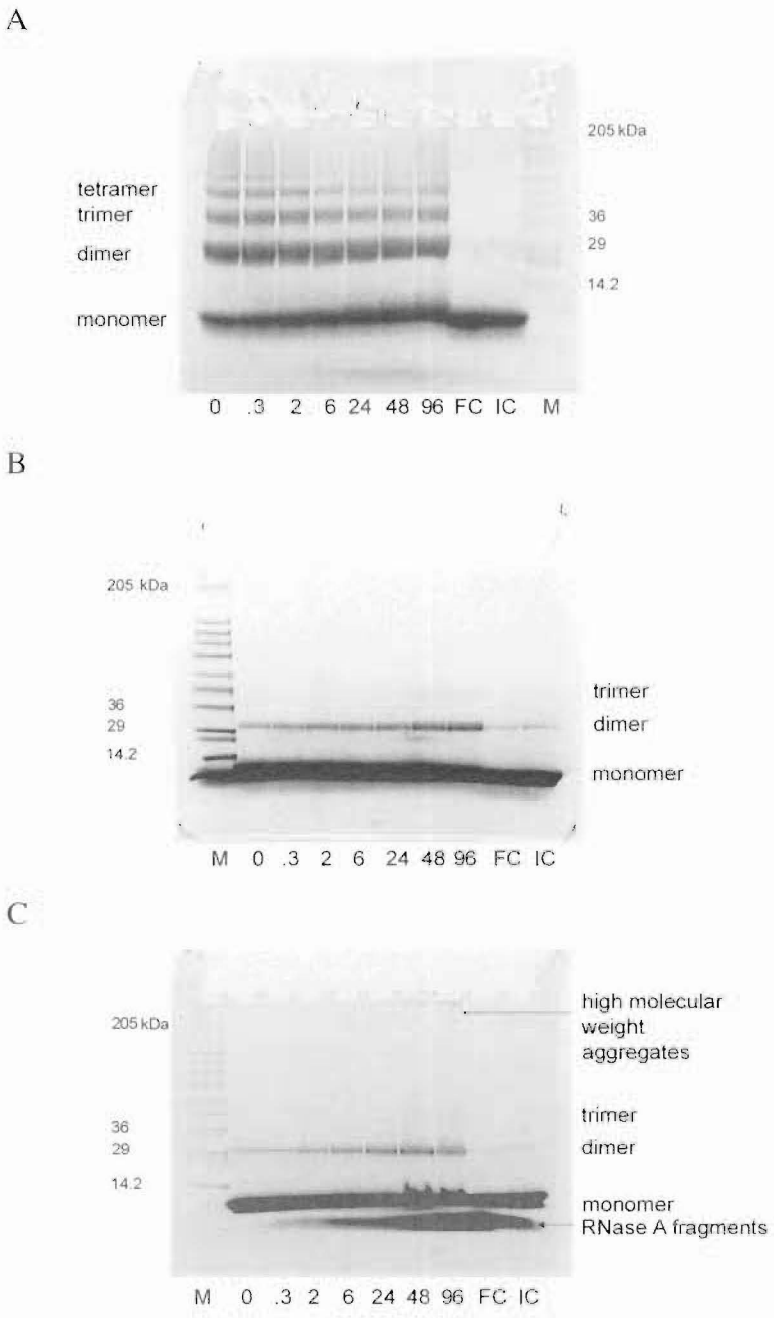


Figure 2.17: 8-16% SDS-PAGE gels of A, methylglyoxal; B, glyoxal C, diacetyl; incubated with RNase A. Abbreviations are as follows: M, wide range marker, 0-96, hours of incubation time of α-dicarbonyl with RNase A at 37°C; FC, RNase A frozen control; IC, RNase A incubated control.

The rate of crosslinking of RNase A by dicarbonyls was as follows: methylglyoxal >> glyoxal ≈ diacetyl, which is in line with results reported by Fayle *et al.* (16), and corroborates the data obtained by monitoring α-dicarbonyls and amine concentration.

2.5.3 The effect of phosphate on the crosslinking reaction

Until this point, phosphate had not been included in any incubations, due to its reported effect on increasing the rate of glycation (98,99). The contribution of phosphate to the extent of crosslinking was examined for work to be undertaken in Chapter five, where a mildly glycated RNase was prepared by incubation with methylglyoxal. This glycated protein was used as a substrate for amadoriase I in order to develop a novel assay to measuring amadoriase I activity. Phosphate was included in these incubations as it was found to stabilise amadoriase I. The addition of phosphate to incubation reactions resulted in different crosslinking rates (Figure 2.18). Inclusion of phosphate in glycation incubations was also shown to make a significant difference in the mean amine concentration compared to those that did not contain phosphate ($P<0.05$) (Appendix one, Table 1). Phosphate also appeared to have a protective effect against the break-down of RNase A.

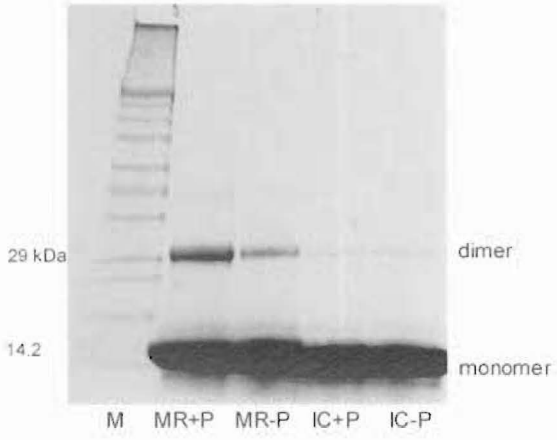


Figure 2.18: 8-16% SDS-PAGE gel comparing the relative rates of RNase A crosslinking in the presence or absence of 100 mM phosphate buffer (pH, 7.4) after 24 hours of incubation. Abbreviations: MR+P, methylglyoxal (10 mM) + RNase A (25 mg/mL) in 100 mM phosphate buffer; MR-P, methylglyoxal + RNase A in the absence of phosphate; IC + P, RNase A incubated control in the presence of phosphate, IC – P, RNase A incubated control in the absence of phosphate.

The only factor to yield a significant difference in the means of lysine concentration was phosphate (Appendix one, Table 1), corroborating the results of the SDS-PAGE analysis of the same incubations (Figure 2.18). An increase in browning in the phosphate-containing incubations relative to those that did not, was also observed.

2.6 General discussion and conclusions

The *o*PDA assay that was undertaken to quantify the concentration of α-dicarbonyls has its merits such as its sensitivity, reproducibility, simplicity and negligible interference. However, the properties of the α-dicarbonyls themselves meant that this assay could not be used as a measure of the extent of the Maillard reaction. As stated previously in section 2.3, α-dicarbonyls decreased in concentration over time only in the presence of water, indicating the α-dicarbonyls (such as methylglyoxal and diacetyl) were reacting with themselves and/or the water to give other products. Figure 2.19 represents what may be occurring in the incubations that led to the observation of a decrease in α-dicarbonyl concentration in the absence of amine.

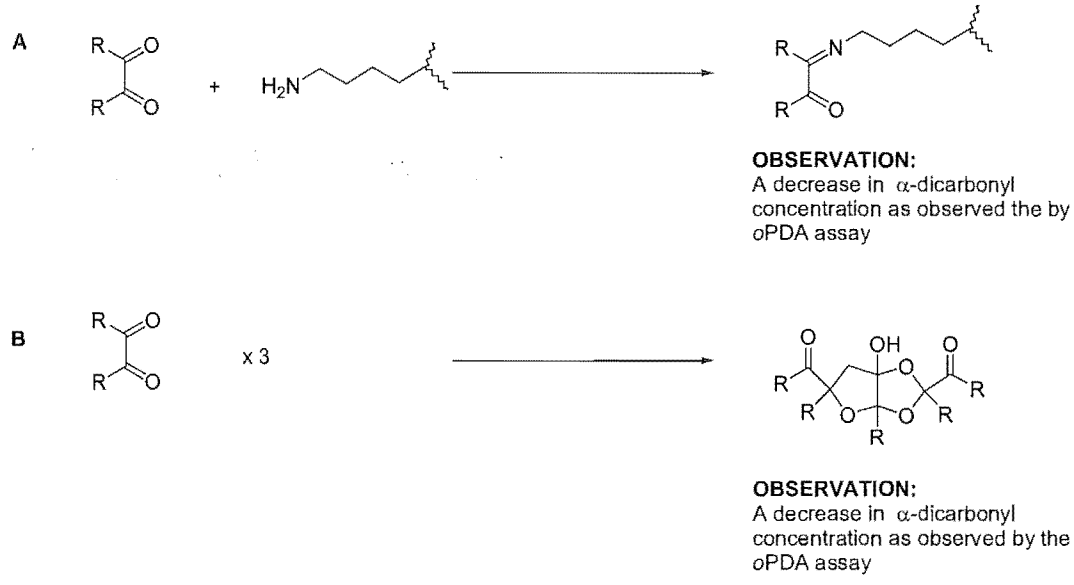


Figure 2.19: A decrease in α-dicarbonyl concentration in amine-containing incubations may be due to two competing processes A, glycation or B, self-condensation (56).

α-Dicarbonyl compounds are known to be highly reactive molecules and thus may be participating in a number of reactions, including the Maillard reaction. To what extent these pathways operate with respect to one another under different conditions (*e.g.* Maillard reaction *vs.* self-condensation of α-dicarbonyl compounds) is largely unknown. This work has shown a decrease in α-dicarbonyl concentration, can be due to the Maillard reaction, but in some cases is closely matched with a decrease through other pathways. Assays that have been employed to measure the generation of α-

dicarbonyl compounds allow for the quantification of α-dicarbonyls over a short period of time. Unlike the approach taken here, these do not encounter problems with measuring subtle decreases in α-dicarbonyl concentration in a solution containing a large amount of unreacted α-dicarbonyl (23,25). This presented a difficulty with the approach undertaken here where the difference between α-dicarbonyl concentration in the presence and absence of RNase can sometimes be very small. Thus, concluding that a certain percentage of α-dicarbonyl drop is due to the Maillard reaction can be difficult. This assay may be more useful for more stable sugars which will be slower to undergo self-condensation.

As the attempts at measuring the extent of the Maillard reaction through measuring α-dicarbonyl concentration proved unsuccessful, attention was shifted toward the other reactant, amine in the form of lysine. A number of researchers have placed emphasis on lysine as a critical reagent in Maillard chemistry, where studies have been undertaken in systems that involve only lysine and a carbonyl compound (100-103). Introduction of protein to the equation allows for the simultaneous study of a number of amino acids in terms of their contribution to the Maillard reaction. The results detailed here, successfully showed that reaction of methylglyoxal with protein showed a drop in amine concentration, with glyoxal and diacetyl showing less reactivity toward amine. This process seems to be offset by the break-down of RNase A over time, thus perhaps resulting in an underestimation of the true rate of amine loss. However, both α-dicarbonyl and lysine studies showed that methylglyoxal was much more reactive than glyoxal and diacetyl, which had similar reactivities.

The crosslinking results obtained in this chapter successfully provided further information with respect to the extent of the protein crosslinking *via* the Maillard reaction, and a system for measuring the specificity of this process by the three α-dicarbonyls. Methylglyoxal was found to crosslink RNase A extensively in a very short period of time and at a much faster rate than glyoxal or diacetyl. The lysine and crosslinking results suggest that in the presence of methylglyoxal, lysine is consumed in the crosslinking reaction, as lysine loss appears to parallel the crosslinking pattern. Lysine loss in diacetyl and glyoxal-treated samples appears to be much less prominent, as was mirrored by a less extensive crosslinking pattern than observed

with methylglyoxal. However, as minimal lysine modification of RNase A was noted in glyoxal-treated samples, some crosslinking was still observed, which may suggest crosslinking of protein *via* amino acids other than lysine. The results were surprising for glyoxal, which has been found to react with lysine to form crosslinks such as GOLD and GOLA (2,3). The reasons why methylglyoxal reacted the fastest of the three α-dicarbonyls studied are further probed in Chapters three and four.

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Chapter Three

Which amino acids are required for protein crosslinking to occur?

3.1 Introduction

The previous chapter analysed the extent of the Maillard reaction during incubation of RNase A with three α-dicarbonyls, providing an insight into the relative rates of reaction by monitoring the decrease of available lysine residues and the degree of crosslinking that accompanies these changes. This chapter focuses on the role of particular amino acids in the crosslinking process. As detailed in Chapter one, the Maillard reaction has a number of consequences for the food industry and the medical field. For the latter, protein crosslinking is a focal point, due to its presumed consequences for the function of the target protein (1).

Within the extensive literature on the reaction of proteins with α-dicarbonyl compounds, little attention has been paid to the relationship of the structural features of these molecules and their rate of crosslink formation. It has been noted by Fayle *et al.* that methylglyoxal is a highly reactive crosslinker and that this reactivity is not matched by the closely related compound, glyoxal (2). Chapter two confirmed this previous result and extended the study to include diacetyl, a structurally similar α-dicarbonyl. It is intriguing that such a small structural change can have such dramatic consequences for crosslinking. Thus, this chapter aimed to address the structural features of small α-dicarbonyl compounds that result in effective crosslinking, by examining the reaction of glyoxal, methylglyoxal and diacetyl with defined protein substrates.

Although efforts have been undertaken to address the extent to which particular amino acids participate in the crosslinking process, no systematic study has yet been undertaken of the precise amino acid requirements for reaction to occur. Most research points to reactions involving lysine residues and arginine residues (3), but whether both amino acids are required, or whether the amino acid requirements change for different crosslinking moieties, has not been investigated in detail.

3.2 A summary of amino acid involvement in crosslinking *via* the Maillard reaction

3.2.1 Artifacts and caveats

A common method for examining the Maillard reaction is to employ relatively simple model systems that involve free lysine or arginine and a carbonyl compound (4-6). From the resulting mixture, products are separated and characterised and then attempts are made to identify the newly-discovered AGE from tissue or in food. Whilst this approach has provided a wealth of information in terms of AGE structures and which amino acids are amenable to reaction, it tells little about the rates of glycation when more than one amino acid is present.

Results from previous protein crosslinking studies must be interpreted with caution, since much research involving intact protein in a model system has relied on acid digestion of the protein (7,8). As outlined in Chapter one, acid digestion can result in a change in the molecular composition of the sample under study, due to break-down of AGEs. Reducing these adducts with sodium borohydride, to increase stability, has been reported as a remedy (9); however, this approach can still lead to AGE break-down (10). Although new methodologies, such as enzyme digests and alkaline hydrolysis have aided the isolation process, the problem has not been entirely solved (3).

To circumvent the problems associated with the analysis of a complex mixture of products, a novel approach to examining the crosslinking phenomenon was taken. A range of starting materials was selected in order to establish precise structural requirements for efficient crosslinking. The reaction of each of the three α-dicarbonyl

compounds with somatostatin, an arginine-free peptide that contains two lysine residues (11), and renin substrate tetradecapeptide, which lacks lysine and contains one arginine (12), along with RNase A, which has both arginine and lysine residues (13), was examined.

3.2.2 Lysine residues

Lysine residues have received the most attention in terms of their participation in the Maillard reaction. In food chemistry, a number of studies have been undertaken that involve reaction of lysine with a variety of sugars (2,14-18). Generally, conditions employed in the food chemistry arena are those that occur under food processing conditions (*e.g.* high pressure and high temperature) and may not be relevant to *in vivo* formation occurring in the human body, although they may have an affect *in vivo* if foods containing these AGEs are ingested (19).

Some of the first crosslinks to be isolated were methylglyoxal lysine dimer (MOLD) and glyoxal lysine dimer (GOLD) (Figure 3.1), from the reaction of protected lysine with methylglyoxal and glyoxal respectively (6). Since their discovery, they have been isolated from human lens proteins (6), some foods (20) and human serum (21). A number of other crosslinks are formed on reaction of lysine with carbonyl compounds, ranging from pentoses to α-dicarbonyls. These include threosidine (22), $PenK_2$ (23), vesperlysine A (LM-1) (24), GOLA (25), Lys-hydroxy-triosidine (26) (Figure 3.1), some of which have been isolated from human tissue (25,27).

Studies with a no-lysine mutant of α-crystallin revealed that crosslinking was observed on treatment with a number of sugars, but to a much lesser extent than the wild type, supporting the conjecture that lysine is an important residue in crosslinking reactions (28). Further, incubation of the no-lysine mutant with another lysine-rich *N*-protected protein showed that crosslinking could occur between lysine and non-lysine residues. Moreover, results from this study suggested arginine could not crosslink with another arginine residue and that a histidine residue was not able to form a crosslink with another histidine residue (28).

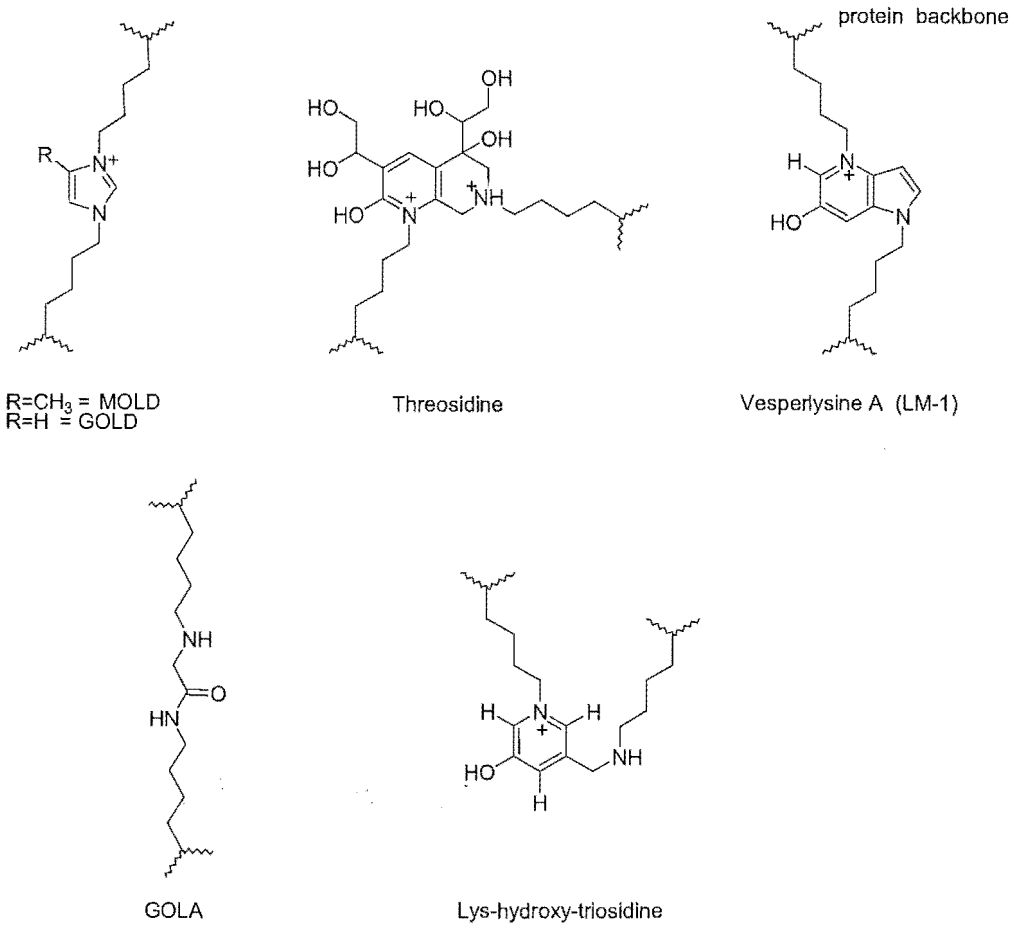


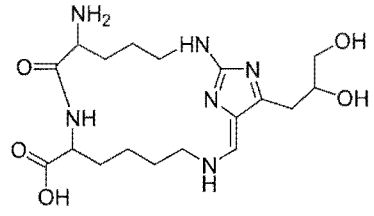
Figure 3.1: A selection of Lys-Lys crosslinks (6,22,24-26). Abbreviations: MOLD, methylglyoxal lysine dimer; GOLD, glyoxal lysine dimer; GOLA, N^6-{2-[(5-amino-5-carboxypentyl)amino]-2-oxoethyl}lysine.

The role of oxygen is important for directing the extent of crosslinking and amino acid specificity. Fu *et al.* found that crosslinking was dependent on the presence of oxygen (29). Under oxidising conditions, lysine and arginine were modified; however, under non-oxidative conditions, a similar loss in lysine was observed, but not arginine (29). From this study, it was concluded that lysine and arginine are important for the crosslinking process to occur, and that their consumption *via* the Maillard reaction varies with respect to oxygen availability (29).

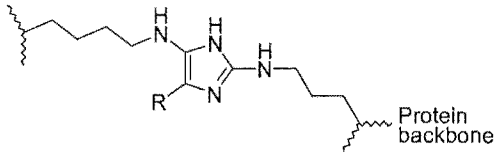
3.2.3 Arginine residues

Along with lysine, arginine residues have been quantified to measure the extent of the Maillard reaction (30,31). A decrease in arginine residues during crosslinking has been reported in a number of studies (8,32-34). As more crosslinks are identified, it is

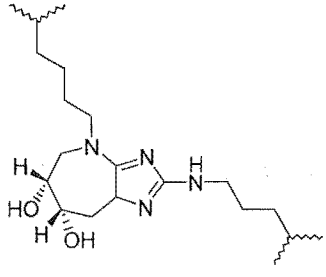
evident that a number of these involve both lysine and arginine residues. Lys-Arg crosslinks elucidated to date include pentosidine (35), Arg-Lys imidazole (ALI) (36), DOGDIC, MODIC, GODIC, glucosepane (37,38) and Arg-hydroxy-triosidine (26) (Figure 3.2).



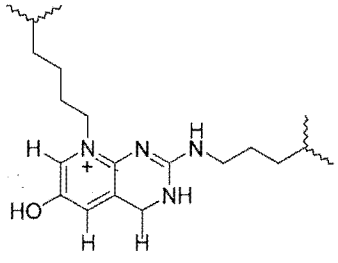
ALI



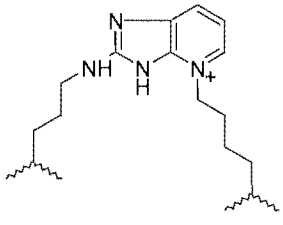
R = H = GODIC
R = CH_3 = MODIC
R = $CH_2(CHOH)_2CH_2OH$ = DOGDIC



Glucosepane



Arg-hydroxy-triosidine



Pentosidine

Figure 3.2: A selection of Lys-Arg crosslinks (26,35-38). Abbreviations: ALI, arginine-lysine imidazole; GODIC, N^6-(2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate; MODIC, N^6-(2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5 methyl-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate[1]; DOGDIC, N^6-{2-{[(4*S*)-4-ammonio-5-oxido-5-oxopentyl]amino}-5[(*2S,3R*)-2,3,4-trihydroxybutyl]-3,5-dihydro-4*H*-imidazol-4-ylidene)-L-lysinate.

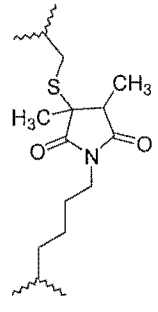
As for the Lys-Lys crosslinks, some Lys-Arg crosslinks from Figure 3.2 have been isolated in human tissue (36,39) and foodstuffs (20). However, one study reported little evidence of major lysine-arginine crosslinks in model studies with protected lysine and arginine residues (40).

[1] The tautomer of MODIC depicted in Figure 3.2 is not that which is generally stated in the literature. The literature tautomer, where the Nε of lysine exists as an imine, is regarded as a less favourable conformation (Prof. Peter J. Steel – Personal communication).

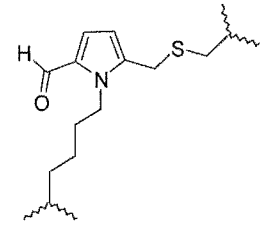
Arginine consumption *via* the Maillard reaction also results in non-crosslinking modifications. Methylglyoxal is reported to react preferentially with arginine, in some cases forming non-crosslinking AGEs (8,10,30). However, methods of quantification can lead to dramatically differing results in terms of arginine modification, which authors attribute to artifacts such as the break-down of acid–labile AGEs that contain arginine during analysis, resulting in overestimation of arginine (30).

3.2.4 Other amino acid residues

Cysteine is a critical residue responsible for maintaining tertiary and sometimes quaternary structure of a protein *via* disulfide linkages. When two protein-bound, reduced cysteine residues are in close proximity and become oxidized, a disulfide linkage is formed between the two residues. With respect to cysteine's role in crosslinking *via* the Maillard reaction, little is known. Cysteine has received more attention in the food arena, where volatile Maillard reaction products formed on reaction sugars have been studied (41). One study has reported extensive loss of cysteine residues during the Maillard reaction (42), and two crosslinks have been proposed that involve cysteine and lysine residues (43,44) (Figure 3.3). Reversible modification of cysteine to form a hemithioacetal in protein has been noted elsewhere, although these modifications do not appear to contribute to crosslinking (30).



Tressl's putative crosslink



Pyrraline-Cysteine crosslink

Figure 3.3: Proposed protein crosslinks involving cysteine (43,44).

Tryptophan has received some attention in the food industry, where the reaction of the free amino terminus with glucose has been reported under food processing conditions (45). Tryptophan residues have been reported to be extensively modified during the

crosslinking of lysozyme by glucose, with glyoxal the only α-dicarbonyl shown to crosslink lysozyme (33). Interestingly, a study from the same workers reported that tryptophan residues of lysozyme were extensively modified by methylglyoxal, glyoxal and diacetyl, but suggested that lysine and arginine residues were more critical to crosslinking (46). Tryptophan participation in crosslinking has been reported elsewhere, but this method could not differentiate between radical crosslinking and crosslinking by electrophilic addition to amine (42).

Early work by Slight *et al.* reported that a loss of arginine and histidine residues on lens crystallin crosslinking could in part involve Lys-His crosslinks (34). A decrease in histidine residues during crosslinking has been reported in other studies (42). To the author's knowledge, the structure of either a tryptophan or histidine-containing crosslink has not, as yet, been reported.

3.3 Investigation of specific amino acid requirements for protein crosslinking involving α-dicarbonyls

The weight of the protein crosslinking literature suggested that lysine and arginine were the main crosslinking participants and thus the role of these two residues was the focus of the crosslinking studies detailed in the remainder of this chapter. To study the role of these two residues, an arginine-containing, lysine-free; and a lysine-containing, arginine-free peptide; and a protein containing both lysine and arginine residues were used.

3.3.1 Reaction of somatostatin with α-dicarbonyls

After an extensive literature search for an arginine-free protein that was readily available, somatostatin was selected. The sequence of the tetradecamer is as follows (11):

Ala-Gly-Cys-**Lys**-Asn-Phe-Phe-Trp-**Lys**-Thr-Phe-Thr-Ser-Cys.

Somatostatin has a number of functions, including inhibition of the release of growth hormone and thyrotropin from the pituitary, along with inhibiting the secretion of insulin (47), glucagon and gastrin (48). Somatostatin (25 mg/mL, 30.6 mM lysine

residue equivalents) was incubated with each of the three α-dicarbonyl compounds at a concentration of 130 mM. The high concentration of α-dicarbonyl was selected in order to achieve crosslinking in a satisfactory period of time, without significant protein degradation.

Methylglyoxal was shown to crosslink somatostatin, as determined by 1-D SDS-PAGE analysis (Figure 3.4). During the course of the incubation, the solution showed substantial browning. By day 3, a large amount of crosslinking occurred, manifesting as smearing on the gel. By day 7, the aggregates formed *via* the Maillard reaction were too large to enter the gel and remained in the wells. Crosslinking was not observed in the controls. Glyoxal and diacetyl, however, did not appear to exhibit the capacity to crosslink somatostatin on this timescale (Figure 3.5 and 3.6 respectively). In contrast to the methylglyoxal incubations, those containing glyoxal and diacetyl-treated somatostatin remained colourless.

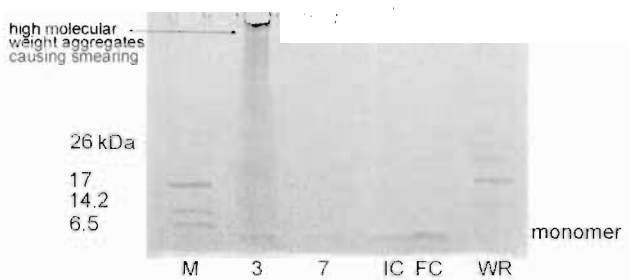


Figure 3.4: 4-20% SDS-PAGE gel of somatostatin following glycation by methylglyoxal. From left to right; M, ultra low molecular weight marker; 3, day 3 incubation of methylglyoxal with somatostatin; 7, day 7 incubation of methylglyoxal with somatostatin; IC, somatostatin incubated control; FC, somatostatin frozen control; WR, wide range marker.

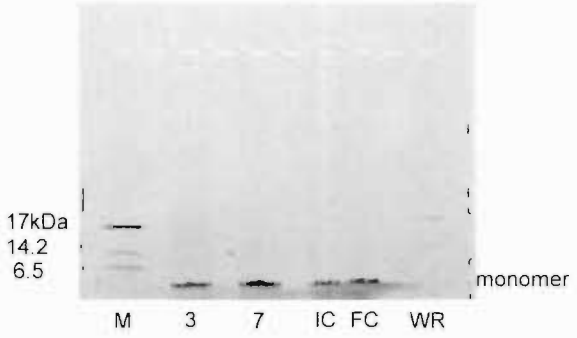


Figure 3.5: 4-20% SDS-PAGE gel of somatostatin following glycation by glyoxal. Abbreviations are as for Figure 3.4.

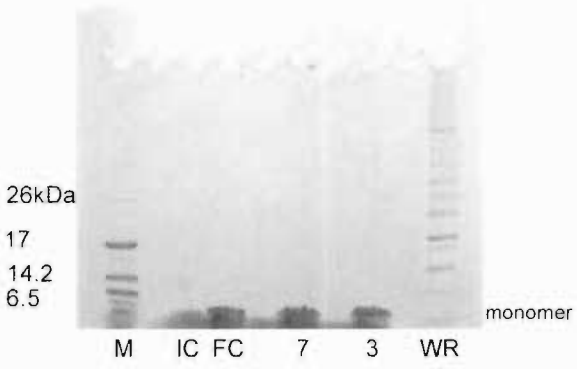


Figure 3.6: 4-20% SDS-PAGE gel of somatostatin following glycation by diacetyl. Abbreviations are as for Figure 3.4.

Thus, under these conditions, methylglyoxal is able to crosslink proteins in the absence of arginine, but glyoxal and diacetyl are not.

3.3.2 Reaction of renin substrate tetradecapeptide (RST) with α-dicarbonyls

The arginine-containing, lysine-free protein selected was renin substrate tetradecapeptide (RST), which was commercially prepared by enzymatic cleavage of angiotensinogen. Its sequence is as follows:

Asp-**Arg**-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser (12)

This peptide is a critical intermediate in the renin-angiotensin system, which is thought to play an important role in the regulation of blood pressure and the volume and composition of extracellular fluid (49). RST was incubated with α-dicarbonyls

under the same conditions employed for the somatostatin incubations (25 mg/mL RST, 14 mM arginine residue equivalents).

The only incubations to exhibit evidence of crosslinking on a 1-D SDS-PAGE gel were those that had been incubated with glyoxal (Figure 3.7).

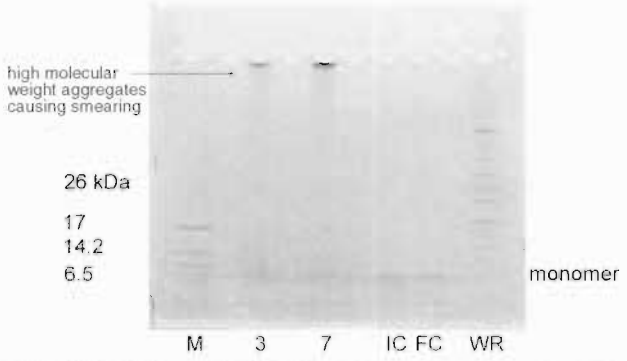


Figure 3.7: 4-20% SDS-PAGE gel of RST following glycation by glyoxal. From left to right; M, ultra low molecular weight marker; 3, day 3 incubation of glyoxal with RST; 7, day 7 incubation of glyoxal with RST; IC, RST incubated control; FC, RST frozen control; WR, wide range marker.

It was hypothesised that the crosslinking observed in the glyoxal incubation may not be entirely due to the arginine residue, but rather, the *N*-terminus. This theory was tested by repeating the incubations with *N*-acetylated RST (Figure 3.8). It was evident that crosslinking had not occurred, thus, the *N*-terminus is essential for crosslinking to occur under the conditions employed in this study.

Figure 3.8: 4-20% SDS-PAGE gel of *N*-protected RST following glycation by glyoxal. Abbreviations are as for Figure 3.7.

Interestingly, neither the methylglyoxal-treated RST, nor that treated with diacetyl exhibited any degree of crosslinking (Figure 3.9 and 3.10 respectively). All three incubations showed some degree of browning, with glyoxal and diacetyl producing darker colours than the methylglyoxal incubations. Comparison to the protein-free controls suggested that this browning could not be accounted for by the α-dicarbonyl itself browning over time.

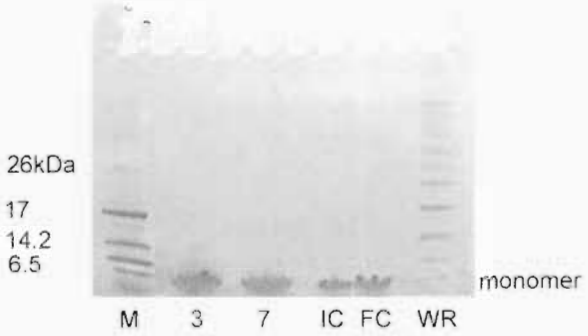


Figure 3.9: 4-20% SDS-PAGE gel of RST following glycation by methylglyoxal. Abbreviations are as for Figure 3.7.

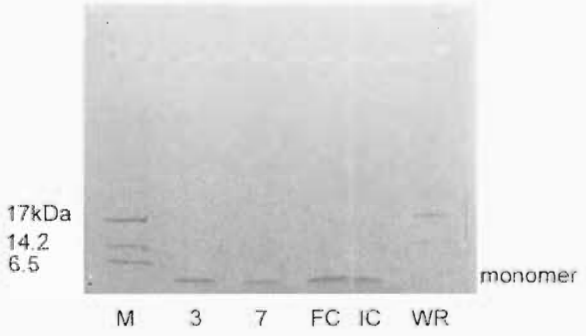


Figure 3.10: 4-20% SDS-PAGE gel of RST following glycation by diacetyl. Abbreviations are as for Figure 3.7.

Thus, in contrast to the results obtained for somatostatin, glyoxal, under these conditions, is able to crosslink protein in the absence of lysine, *via* the *N*-terminus, whereas methylglyoxal and diacetyl are not. The presence of arginine alone was not sufficient to allow crosslinking for any of the compounds tested. This is, to the author's knowledge, the first time that such specificity in structural requirements for protein crosslinking by α-dicarbonyls has been demonstrated.

3.3.3 Comparative crosslinking ability with RNase A

As RNase A contains both lysine and arginine residues within its sequence, it was reacted with the same series of α-dicarbonyls to determine the relative crosslinking rates in the presence of both amino acids. Figure 3.11 shows a typical result depicting the extent to which RNase A was crosslinked by methylglyoxal, glyoxal and diacetyl.

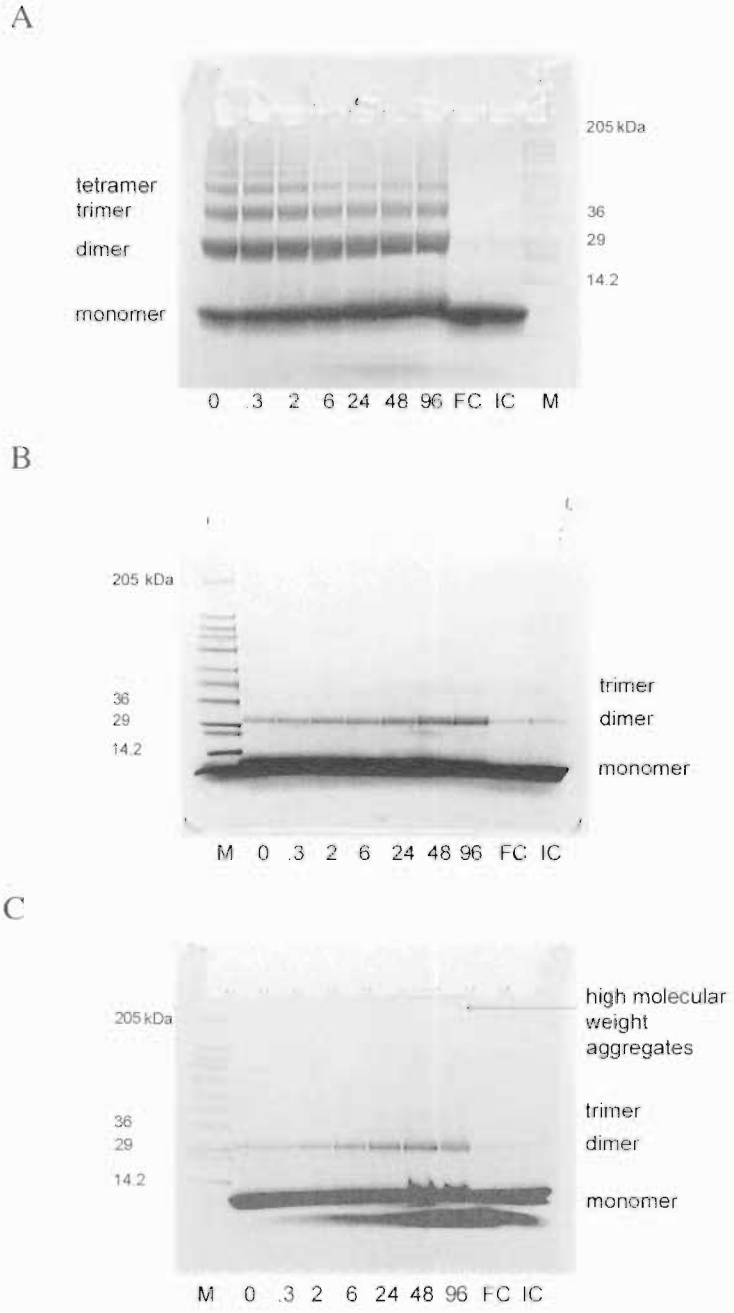


Figure 3.11: 8-16% SDS-PAGE gels of A, methylglyoxal; B, glyoxal; C, diacetyl; incubated with RNase A. Abbreviations are as follows: M, wide range marker, 0-96, hours of incubation time of dicarbonyl with RNase A at 37°C; FC, RNase A frozen control; IC, RNase A incubated control. See also Figure 2.17.

The rate of crosslinking of RNase A by dicarbonyls was as follows: methylglyoxal >> glyoxal ≈ diacetyl, which is in line with results reported by Fayle *et al.* (2). Other workers have also reported that methylglyoxal appears to extensively crosslink protein in a short time-frame (8,33). However, the results reported here are in contrast to a study undertaken by Kato *et al.* where the reactivity of crosslinking was as follows: diacetyl > glyoxal > methylglyoxal (46). The authors state that the extent of crosslinking was related to the extent of browning observed, which contrasts to results detailed here. Kato *et al.* used lysozyme, which contains 6 lysine and 11 arginine residues (50), and crosslinking reactions were undertaken in the presence of phosphate at 50°C. An increase in temperature will increase the rate of the Maillard reaction (51), along with the addition of phosphate to incubations (Chapter two) (52,53). These factors may help to explain the difference in crosslinking. The differing lysine and arginine content between proteins (RNase A; 10 Lys, 4 Arg) may result in differing reaction pathways for the α-dicarbonyls, due to amino acid limitation, and may be manifested as different crosslinking rates.

Interestingly, the RNase A formed insoluble masses immediately on incubation with methylglyoxal. Formation of such aggregates can be indicative of amyloid fibril formation and thus, the samples were tested for the presence of fibrils. Recently, it has been shown that a number of proteins have the capacity to form fibrils and that it may be a generic process within many proteins (54), thus, RNase A may be capable of forming fibrils on glycation. Fibril formation as a result of glycation has been reported on incubation of insulin with methylglyoxal (55), and on incubation of bovine serum albumin with either glucose, fructose or glyceraldehydes but, interestingly, not with glyoxylate (56).

The presence of fibrils was measured using a Congo red dye, which binds to the ordered structure of the fibril. When viewed under a polarising light microscope the fibrils exhibit a green birefringence (57). This was not observed for the methylglyoxal + RNase A samples, but was with authentic, insulin fibrils that were prepared as a positive control. A second test was undertaken to confirm this using another amyloid-specific dye, Thioflavin T. Upon binding of fibrils, an increase in the excitation spectrum at 450 nm, and an enhanced emission spectrum at

approximately 482 nm are observed (58). Unlike the insulin fibril controls, a shift at the appropriate wavelengths was not observed in the methylglyoxal + RNase A samples on incubation with Thioflavin T. Thus, the aggregates isolated in this work may be non-fibrillar in nature and form amorphous aggregates that have been reported in previous glycation experiments (56). To the author's knowledge fibril formation has not been reported in RNase A, which is generally noted for its stability.

3.4 General discussion and conclusions

The fact that glyoxal did not appear to form any crosslinks in the lysine-containing peptide may be due to the predominance of non-crosslinking AGEs, such as CML (Figure 3.12). Indeed, it has been reported that on reaction of lysine with glyoxal, the non-crosslinking CML was present in much greater quantities over crosslinking AGEs *e.g.* GOLA and GOLD (25). This is consistent with the results reported in this chapter.

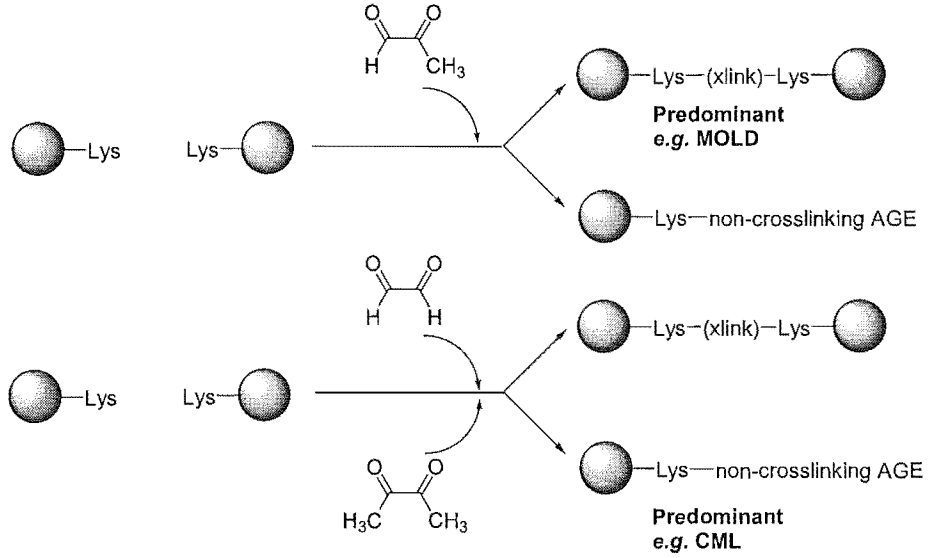


Figure 3.12: Addition of methylglyoxal to an arginine-free protein may result in the predominance of crosslinks. Addition of glyoxal or diacetyl to an arginine-free protein may result in the predominance of non-crosslinking AGEs. Spheres represent the remainder of the protein. For clarity, *N*-terminus has been omitted, but could be interchanged with above Lys.

In the absence of lysine, crosslinking by methylglyoxal was not observed. This is consistent with the consensus that methylglyoxal reacts preferentially with arginine

over lysine (8,30), if these are arginine modifications that do not lead to crosslinking, such as argpyrimidine, carboxymethylarginine and tetra-hydropyrimidine and imidazolone compounds (Figure 3.13) (4,59). This may account for the browning observed in non-crosslinking samples such as incubations of glyoxal and diacetyl with RST.

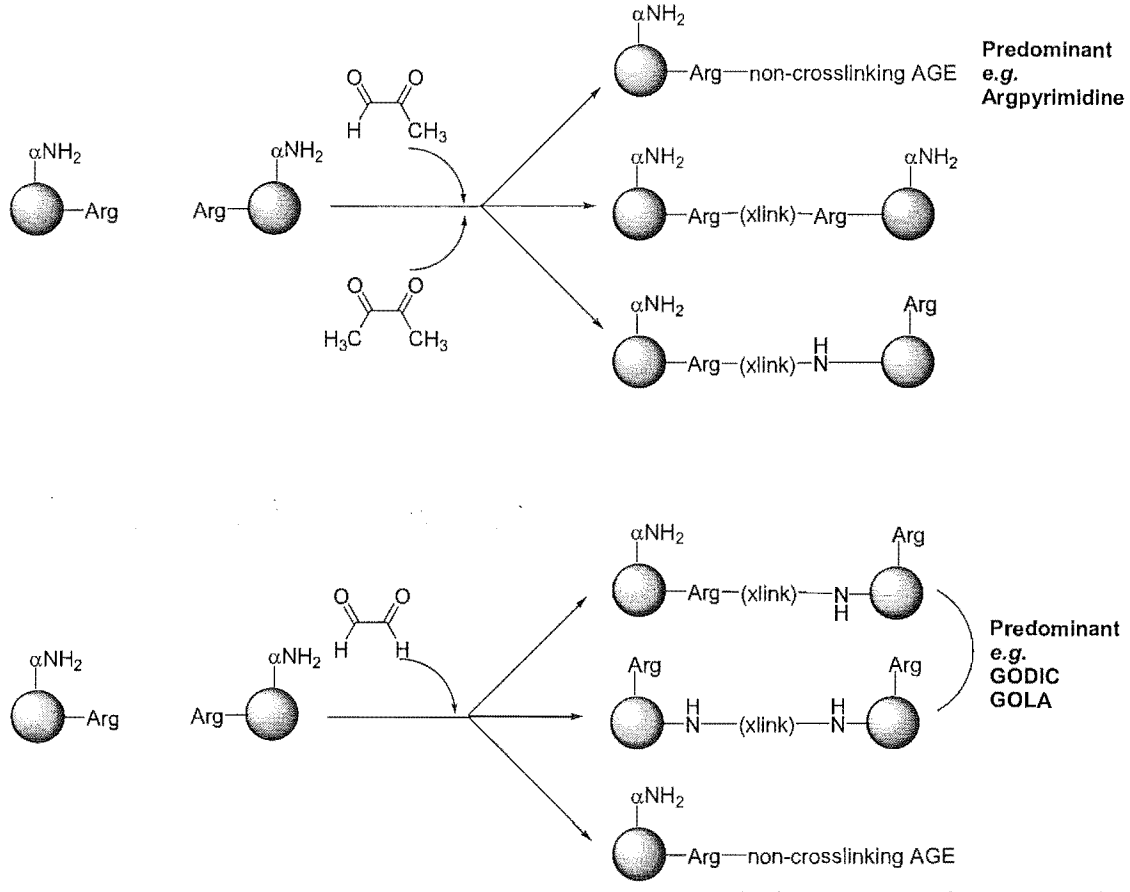


Figure 3.13: Methylglyoxal and diacetyl reaction with a lysine free protein may result in the predominance of a non-crosslinking AGE. Glyoxal, on the other hand, appears to favour the formation of a crosslinking AGE. Spheres represent the remainder of the protein.

To the author's knowledge, there have not been any reports of arginine-arginine crosslinks mediated by methylglyoxal, although one has been reported involving glyoxal, but this requires furan-2-carboxaldehyde (60), not present in the above incubations.

The finding that glyoxal is able to crosslink the lysine-free peptide, *via* the *N*-terminus, whereas methylglyoxal cannot, was not predicted. Presumably, this reflects a subtle balance between competing reaction pathways, and perhaps the rapid reaction

of methylglyoxal with arginine to form argpyrimidine renders it unavailable for the slower crosslinking reaction with the *N*-terminus (Figure 3.13). Glass and Pelzig have reported reversible modification of arginine residues by glyoxal under particular experimental conditions (61). The modification of guanidine, related to the functional moiety of arginine, by glyoxal in aqueous conditions has also been reported Bengelsdorf (62). In the same manner as methylglyoxal-arginine modifications the majority of the glyoxal-mediated arginine modifications are also non-crosslinking in nature. However, if the reaction with glyoxal is more easily reversed than that with methylglyoxal, then this may account for the difference in behaviour between the two systems. Whether the glyoxal crosslink in this system is between the two *N*-termini of RST, or between one *N*-terminus and an arginine residue, is, to date, unclear.

The observation that glyoxal is a less efficient crosslinker of RNase A than methylglyoxal is chemically consistent with the proposed mechanism for imidazolium formation (63). Reaction of methylglyoxal requires a fragmentation reaction (Figure 3.14 step 4) which is predicted to be more favourable than for reaction of glyoxal, due to increased relative stability of the substituted leaving group in step 4. Diacetyl was also observed to be less reactive at crosslinking than methylglyoxal. Diacetyl contains two ketone moieties. Reaction of diacetyl to form an imidazolium crosslink would be hindered, as ketone groups would react more slowly than aldehydes in step 2 (Figure 3.14), due to a combination of steric hindrance and reduced electrophilic reactivity of the carbonyl centres (64).

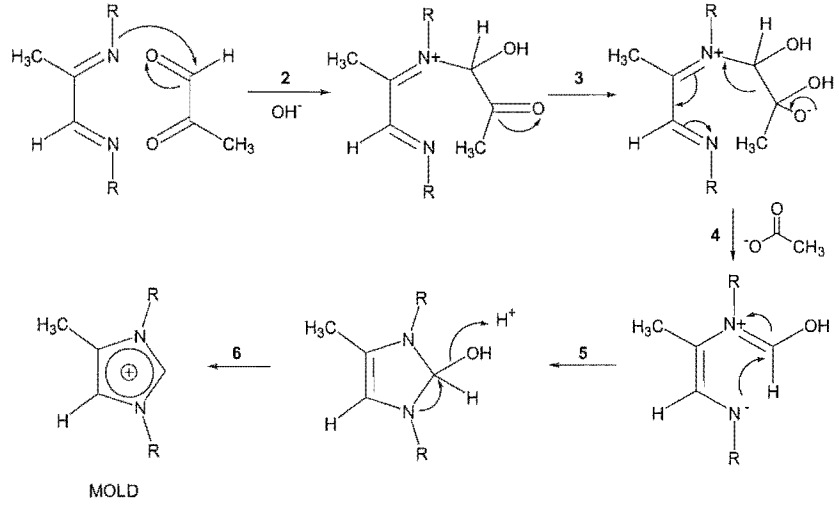


Figure 3.14: Proposed mechanism for formation of MOLD. R, lysine residues (63).

The most striking feature of the results reported in this chapter is perhaps not the relative rates, but the specificity of the reactions observed: small structural differences in the α-dicarbonyl moiety lead to a large change in amino acid requirements.

This structure-activity study leads to two unequivocal conclusions. Firstly, that arginine is not essential for crosslinking to occur with α-dicarbonyls, as evidenced from the work with methylglyoxal and somatostatin. Secondly, that a free amino group, either as the *N*-terminus or as a lysine residue is required for protein crosslinking under these conditions, in this series. It can be further speculated that the presence of both lysine and arginine residues, for example in RNase A, leads to the most efficient crosslinking, but that an excess of arginine residues leads to the predominance of non-crosslinking modifications, such as argpyrimidine.

Perhaps most importantly, this study has shown that results from the crosslinking of one molecule cannot be extrapolated to predict the behaviour of another, however closely related. This is an extremely important observation when designing novel therapeutic strategies based on results from simple model systems in the laboratory.

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Chapter Four

Does protein crosslinking *via* α-dicarbonyls affect enzyme function?

4.1 Introduction

Chapter three began to address the consequences the Maillard reaction had on a target protein by examining protein crosslinking mediated by α-dicarbonyl molecules. Close attention was paid to the amino acid requirements for this process to occur, providing more clues to molecular detail of the Maillard reaction. This chapter further assesses the effect that the Maillard reaction has on a protein, by probing the relationship of protein crosslinking to the effect on the native function of an enzyme.

Therapeutic strategies have been designed to inhibit ageing by preventing or reversing protein crosslinks, making the tacit assumption that loss of function is due to the crosslinking process, and further, that preventing crosslinking will maintain function. This assumption has yet to be rigorously tested. Nevertheless, it forms the basis for the development of a class of Maillard inhibitors that act to break protein crosslinks (1-4). However, these results have been the source of some debate (5,6). Along with the crosslink-breakers, compounds such as aminoguanidine (7), are reported to trap reactive dicarbonyl species (8), sequestering them before they can react with the protein. More recently, it has been speculated that effectiveness of proposed dicarbonyl trapping and crosslink breaking compounds as AGE inhibitors may be due to other characteristics, such as metal ion chelating and radical trapping properties, which may provide an additive effect (9-11). The final section of this chapter addresses the effect of three purported Maillard inhibitors, by assessing their ability to

inhibit crosslinking and maintain native function of the target protein, in order to test the assumption that prevention of crosslinking will preserve protein function.

4.2 Literature regarding the native function of a protein during the Maillard reaction.

As enzymes play a key role in cellular function, an understanding of the effect of the glycation process on a protein is critical (10,12,13). Glycation of RNase A by methylglyoxal and glyoxal reduces native activity of the protein to 45% and 50% respectively, after one day of incubation in 200 mM MOPS buffer, with a dicarbonyl:protein ratio of 57:1 for methyglyoxal and 45:1 for glyoxal (14). Further studies with glucose have shown a 42% inactivation per mol of glucose per day, which preceded lysine loss (12). An active site lysine, Lys-41, was found to be preferentially modified by glucose to yield a catalytically deficient enzyme (12). A loss of enzymatic function of cellular homeostasis on glycation, such as glutathione reductase has been shown (13,15,16). Furthermore, modification of critical metabolic and blood clotting enzymes such as glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase has been reported on incubation with methylglyoxal and glyoxal (13,17,18). Glycated proteins themselves may further affect other protein function, as glycated proteins have been shown to inactivate protein that had not previously been exposed to sugar (13). The formation of covalently crosslinked human interferon during over-expression in bacterial systems has been reported. Following crosslinking, the interferon has been shown to lose its native antiviral activity (19).

Although glycation may pose a serious threat to protein function, two reports have shown otherwise. Recent work studying glycation of heat shock protein-27 (HSP-27) showed that modification by methylglyoxal can protect against cell death through oligomerisation of HSP-27 (20). In other work, the modification of α-crystallins by methylglyoxal, leading to arginine modification, results in a paradoxical enhancement in the chaperone function of these proteins (21). This later finding is thought to have evolved in the eye lens as a protective measure against age-related protein modifications.

Where crosslinking studies regarding biological function are concerned, the majority of the literature has focused on crosslinking of structural proteins such as collagen and lens crystallins (22-25). Crosslinking of lens crystallins by sugar has been studied closely by many workers due to its slow turnover, and thus high content of AGEs (26). It is thought that crosslinking leads to a change in tertiary structure of lens crystallins, insolubilisation of protein and the gradual formation of cataracts (27,28). Loss of chaperone ability of α-crystallins has been reported on incubation with methylglyoxal and glyoxal (29,30). Changes in other areas in the eye including the vitreous, cornea and retina have also been associated with AGEs (27). Glycation of some key elements of collagen may affect collagen IV assembly and matrix-cell interactions *in vivo* (31,32). In collagen studies, it has been reported that loss of function cannot be solely accounted for by crosslinking, as only modification of a critical lysine residue is required (32). Crosslinking of collagen by AGEs has been reported to decrease collagen digestibility, increase stiffness in the collagen network of cartilage and induce an expansion in molecular packing which can affect movement of fluid across arteries (23,32,33). Little is known about the molecular detail of the major crosslinks responsible for the effects observed in crosslinked collagen (34).

In summary, the crosslinking – function relationship has not been extensively probed. Thus, the following section explores this relationship by examining the effect that crosslinking had on the native function of a model protein, RNase A.

4.3 Conditions employed to probe the crosslinking-function relationship

In order to fully assess the effect of crosslinking on function of protein, a number of areas needed to be addressed, including selection of an appropriate protein and development of an assay to measure its activity following glycation. This section details both of these areas along with the inclusion of Maillard inhibitors to gain further information with respect to crosslinking and activity.

4.3.1 Ribonuclease A catalytic mechanism

The first step in probing the crosslinking – function relationship, was to select a model protein. RNase A was viewed as an excellent candidate for these studies due to reasons outlined in Chapter two, such as stability. Also, as enzymatic activity was under study, an added advantage was that the catalytic mechanism and amino acids involved in this process have been extensively studied. RNase A acts to cleave ribonucleic acid (RNA) at the P-$O^{5'}$ bond. It is thought that this occurs *via* a transphosphorylation (Figure 4.1 **A**) and hydrolysis reaction (Figure 4.1 **B**).

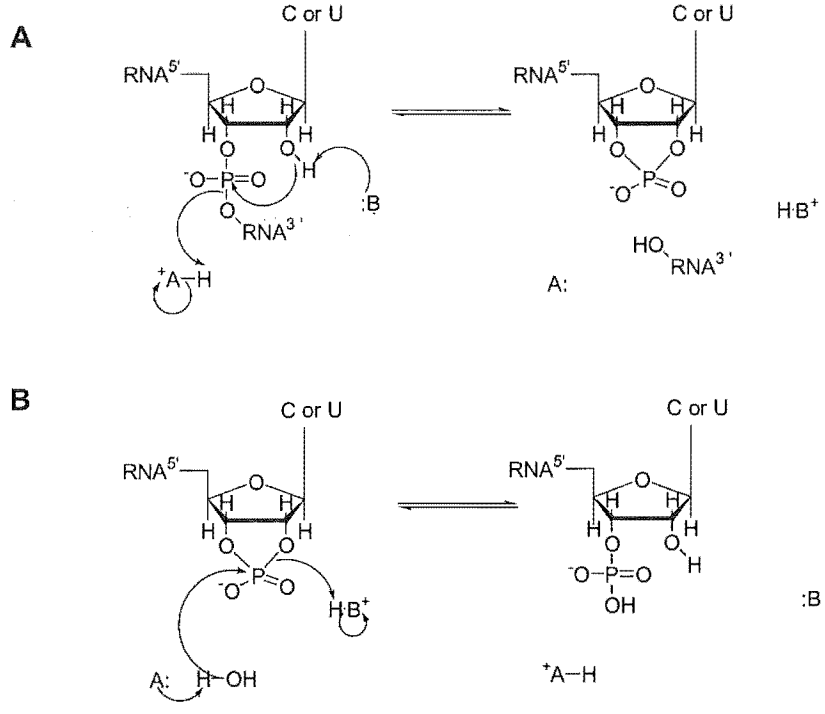


Figure 4.1: Proposed mechanism of RNase A action. **A**, the transphosphorylation step; **B**, the hydrolysis step. A and B represent His12 and His119 respectively (35). Abbreviations: C, cytosine; U, uracil.

RNase A has a number of subsites that contain residues that are essential for correct binding and catalysis of polymeric substrates such as RNA (36). Critical substrate binding and catalytic residues within these subsites are outlined in Figure 4.2, some of which may be amenable to modification by carbonyls, such as Lys7, Arg10, Lys41 and Lys 66, due to their available amine or guanidinium functionality.

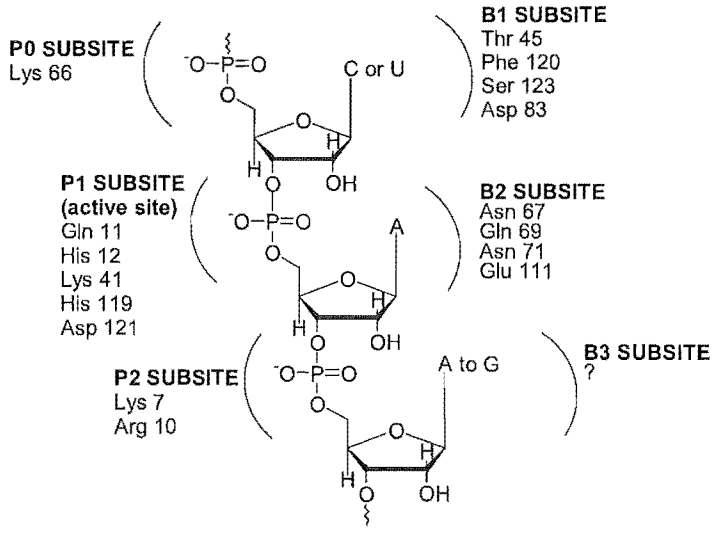


Figure 4.2: The apparent subsites in RNase A and a bound molecule of RNA. P denotes phosphate subsites. B denotes base subsites. For clarity, ribose subsites have not been included. After Raines (37), with additional information from Leonidas *et al.* (38) and Nouges *et al.* (36). Abbreviations: A, adenine; G, guanine; C, cytosine; U, uracil. ? represents the fact that the residues of subsite B3 are not established.

4.3.2 Review of existing methods to measure ribonuclease A activity

Having selected RNase A as the protein for subsequent studies, a suitable RNase A assay was selected to assess the catalytic ability of RNase A following glycation by α-dicarbonyl compounds. As RNase A has been extensively studied, a number of options were available, some of which are detailed below.

Studies assessing activity of RNase A have employed either its natural substrate RNA (39-41), a cyclic monophosphate (14,42), synthetic polynucleotides (43), or fluorogenic substrates (35). Assays generally measure either the rate of generation of nucleosides (43), or nucleotides (40,44). Some of these assays require estimation of initial rate due to end-point assay measurements, which can lead to over- or under-estimation of initial rate (45). Another disadvantage in some available methodologies, such as those described by Anfinsen *et al.* and modifications thereof, is that they can be very labour and time-intensive (46). These assays have shown differing kinetic parameters depending on the substrate employed (44). In more recent years, measurement of RNase A activity by a fluorescence method has been

undertaken (47,48). When fluorogenic dinucleotide substrates are cleaved by RNase A, a large increase in fluorescence is observed. This can be measured over time and the subsequent data is used to calculate and initial rates (47).

A different approach to standard colourimetric enzyme assays is taken by zymogram assays, which involves the incorporation of RNA into an agarose film, which is then exposed to RNase A in the presence of ethidium bromide, and the progress of RNase A action observed under UV light (312 nm) after electrophoresis. The principle is based on the fact that ethidium bromide fluoresces with only large RNA molecules, but not degraded RNA (49). The advantage of this method is that it is very sensitive, although it is a more qualitative approach, generally employed for detection of different isoforms of RNase A in nature, than other assays mentioned above. (49).

The methylene blue assay is an excellent means for determining RNase activity in a complex mixture (37). It is possible to assay activity of a complex mixture, due to the fact that enzymatic digestion of RNA is measured at 688 nm, out of the absorption range of many biomolecules. The principle of the assay is that as methylene blue intercalates with RNA, a shift in absorbance maximum of the methylene blue monomer from 653 to 688 nm (intercalated) is observed (Figure 4.3) (50). On addition of RNase A, the chelated RNA is digested and the monomeric methylene blue monomer is liberated, causing a shift in absorbance to 653 nm (Figure 4.3).

The assay was selected for a number of reasons. Firstly, the incubations under study were considered as complex mixtures, which this assay is ideally suited for, as they contained α-dicarbonyl compounds, RNase A and their reaction products. Secondly, enzymatic studies using the native substrate of the enzyme may provide more information regarding catalysis over synthetic substrates. Thirdly, the assay requires readily available materials and equipment and requires little preparation. Finally, this method allows the measurement of rate as the reaction progresses, as it is not an end-point assay, and thus allows for a more accurate determination of initial rate.

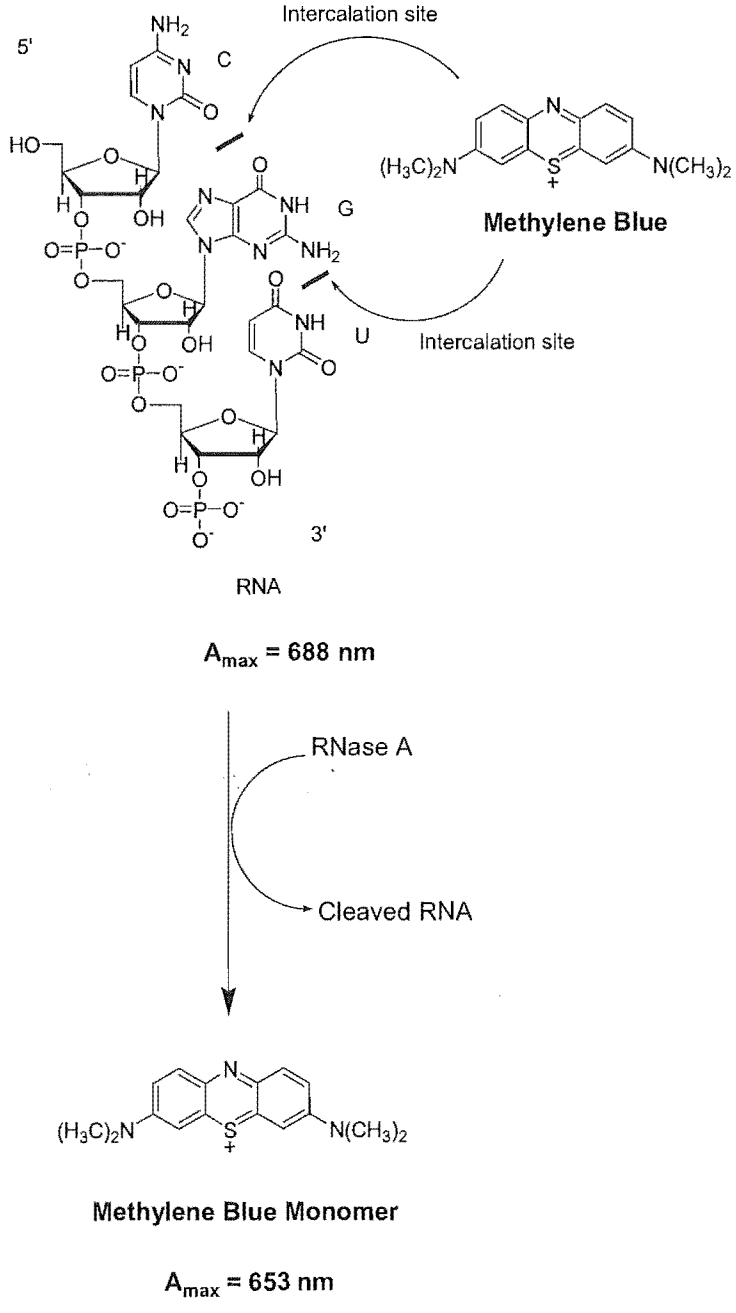
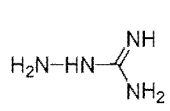


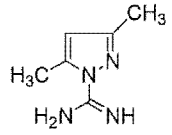
Figure 4.3: Principle of the methylene blue assay. Intercalation of methylene blue into RNA between two adjacent bases results in an A_{max} at 688 nm. Cleavage of this complex by RNase A liberates the methylene blue monomer. Abbreviations: C, cytosine; G, guanine; U, uracil.

4.3.3 Addition of Maillard reaction inhibitors to study the effect of protein crosslinking on native function

In order to establish whether the loss in function that accompanies protein crosslinking was a direct result of the crosslinking process, or a co-incident event, two previously reported crosslinking inhibitors were selected: aminoguanidine and 3, 5-dimethylpyrazole-1-carboxamidine (DMPC) (Figure 4.4).



Aminoguanidine



3,5-Dimethylpyrazole-1-carboxamidine

Figure 4.4: Maillard inhibitors selected to probe the crosslinking-function relationship.

Aminoguanidine is a reported inhibitor of the progression of the Maillard reaction and has been found to be effective in a number of *in vitro* and *in vivo* experiments (7,51,52). The mode of action was initially proposed to be reaction with glucose, rendering the latter unable to react with amines (7), but more recently has been attributed to its ability to react with α-dicarbonyl compounds, such as those used in this study (8). DMPC has also been proposed as a Maillard reaction inhibitor, but is less well-characterised (53). Minimal information has been provided as to the mechanism of crosslinking inhibition by DMPC (53).

4.3.4 Method development of the methylene blue assay to determine RNase A activity

A calibration curve was constructed to determine the RNA concentration to be used for calculation of rate of RNA cleavage. The resultant calibration curve was hyperbolic in nature (Figure 4.5) which is consistent with that reported by Griener-Stoeffele (50).

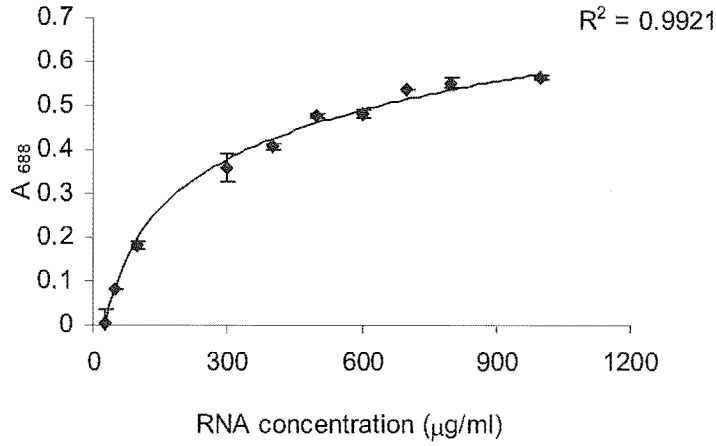


Figure 4.5: Calibration curve of RNA concentration using the methylene blue assay. Each point is an average of three samples with the experiment performed at least in triplicate. Error is expressed as standard error of the mean for triplicate samples.

A concentration of 800 μg/mL RNA was selected for ensuing method development, in line with Greiner-Stoeffle (50). The next step was to determine whether enzyme concentration was proportional to rate of RNA cleavage. The RNase A concentration was varied from 0.05 to 0.4 μg/mL, whilst maintaining a fixed concentration of RNA (800 μg/mL). Analysis of the experiment shows a linear relationship between RNase A concentration and initial rate (Figure 4.6).

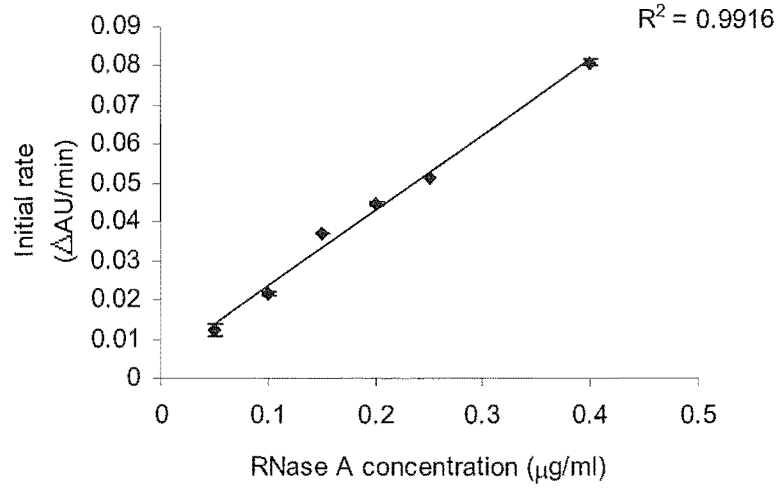


Figure 4.6: Change in absorbance over time on varying RNase A concentration. Each point is an average of two samples with the experiment performed at least in triplicate. Error is expressed as standard error of the mean for duplicate samples.

In an attempt to determine K_m and V_{max}, RNase A was fixed at a selected final assay concentration, 0.15 μg/mL, and the RNA concentration varied. During these experiments a reproducible loss in activity was noted at high concentrations of RNA (Figure 4.7).

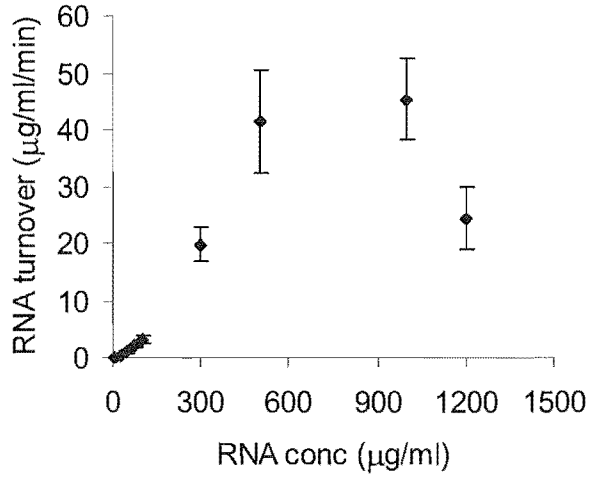


Figure 4.7: Rate of RNA cleavage with varying RNA concentration. RNase A concentration is 0.15 μg/mL. Each point is an average of two samples with the experiment performed at least in triplicate. Error is expressed as standard error of the mean of duplicate samples.

A decrease in activity at high concentration of substrate for some enzymes has been observed (54). Product inhibition can be responsible for this phenomenon, and has been shown in work using RNase A with RNA as a substrate (55). A lower concentration of 600 μg/mL of RNA was selected to overcome this problem and the method was further developed (Figures 4.8 A and B). Final concentrations of 600 μg/mL RNA and 0.15 μg/mL RNase A were used for subsequent experiments undertaken to assess activity of RNase A following crosslinking by α-dicarbonyl.

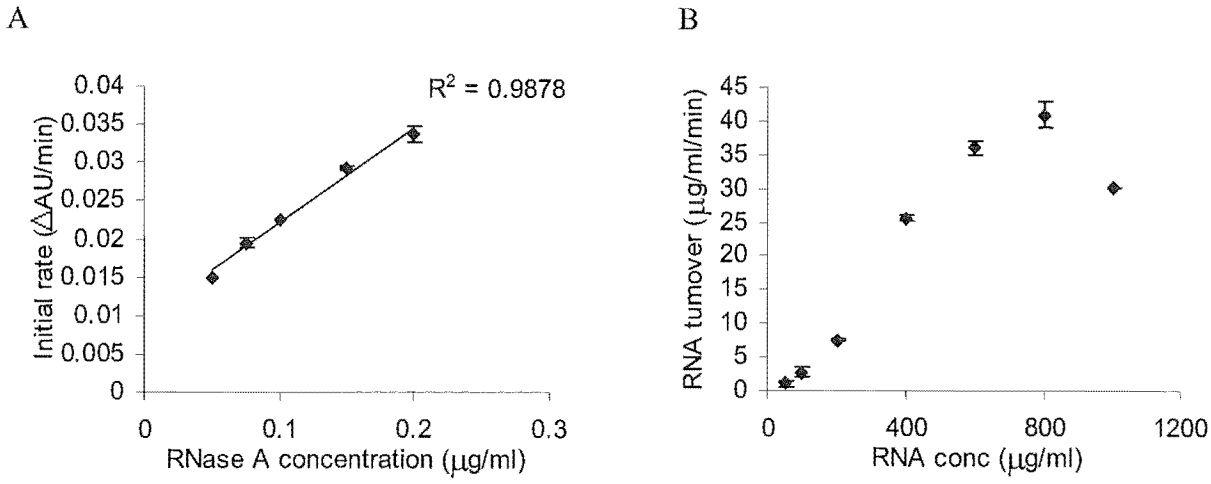


Figure 4.8: A, Change in absorbance over time on varying RNase A concentration. Each point is an average of two samples with the experiment performed at least in triplicate. Error is expressed as standard error of the mean of duplicate samples. B, Rate of RNA cleavage with varying RNA concentration. RNase A concentration is 0.15 μg/mL. Each point is an average of three samples with the experiment performed at least in duplicate. Error is expressed as standard error of the mean of triplicate samples.

With a successful method for quantifying RNase A activity in hand, the effect of α-dicarbonyl compounds and Maillard inhibitors on the catalytic activity of the protein could be determined.

4.4 Analysis of activity and crosslinking of RNase A on incubation with α-dicarbonyl in the presence or absence of Maillard inhibitors

4.4.1 Aminoguanidine

On incubation with methylglyoxal, RNase A was found to exhibit a time dependent drop in activity with almost a total loss of activity after four days (Figure 4.9). RNase A incubated with methylglyoxal in the presence of aminoguanidine was observed to maintain activity over time (Figure 4.9), indicating that aminoguanidine protects the function of RNase A. As described in chapter two, crosslinking appeared to occur immediately after the methylglyoxal was added to RNase A (Figure 4.10 A). A marked inhibition of crosslinking was observed on incubation of RNase A and

methylglyoxal with aminoguanidine (Figure 4.10 B), which is consistent with previous reports (52,56).

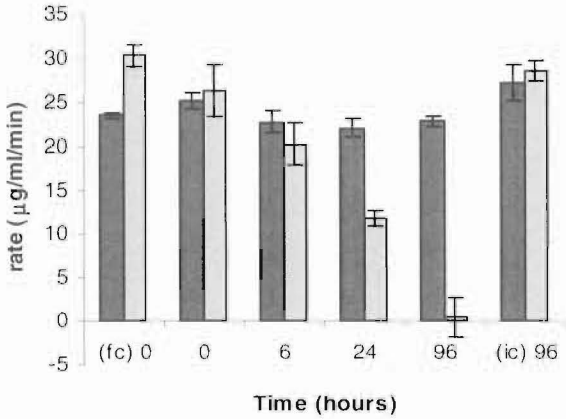


Figure 4.9: RNase A activity over 96 hours following incubation with methylglyoxal in the presence (■), or absence ( ), of aminoguanidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate readings. X axis: FC (0), RNase A frozen control; 0-96, time in hours; IC (96), RNase A incubated control. Rate expressed as µg/mL of RNA cleaved per min.

A B

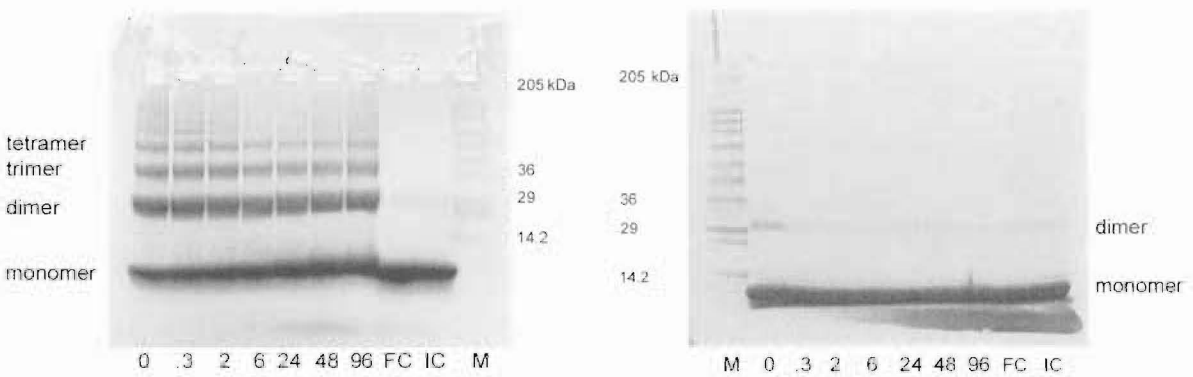


Figure 4.10: 8-16% SDS-PAGE gels of methylglyoxal incubated with RNase A in the absence A, or presence B, of aminoguanidine. Abbreviations are as follows: M, wide range marker; 0-96, hours of incubation time of dicarbonyl with RNase A at 37°C; IC, RNase A incubated control; FC, RNase A frozen control.

Based on these results, crosslinking of RNase A by methylglyoxal corresponds to the loss in activity observed in glycated RNase A as aminoguanidine appears to successfully inhibit crosslinking and native function is retained. However, non-

crosslinking AGEs must also be considered in this process. The decrease in activity is gradual, with crosslinking occurring immediately, as shown in Figure 4.10 A. It may be that aminoguanidine not only inhibits crosslinking, but also non-crosslinking modifications of protein, that may be in part responsible for inhibition of RNase A activity. Results for diacetyl and glyoxal were less dramatic than for methylglyoxal (Table 4.1).

Dicarbonyl	Aminoguanidine		No inhibitor	
	Crosslinking [a]	Rate of reaction (μg RNA/mL/min)[b]	Crosslinking	Rate of reaction (μg RNA/mL/min)
Methylglyoxal	-	22.9 ± 0.4	+++	3.8 ± 1.6
Glyoxal	-	29.3 ± 1.7	+	17.9 ± 0.7
Diacetyl	-	30.2 ± 1.1	+	22.1 ± 0.9
No Dicarbonyl	-	29.5 ± 0.6	-	28.8 ±1.1

Table 4.1: Comparison of RNase A crosslinking with catalytic activity. [a] Positive crosslinking results were those which showed increased crosslinking compared to controls. [b] Rate calculated from methylene blue RNase A activity assay. Errors are ± s.e.m of triplicate readings. Data is that from the 96 hour incubations for each dicarbonyl. [c] Controls values are an average of results yielded for each differing α-dicarbonyl series, error is the standard error of the mean for these readings. Rate expressed as μg/mL of RNA cleaved per min.

In the case of glyoxal, addition of aminoguanidine resulted in the maintenance of RNase A activity, as observed for methylglyoxal (Table 4.1) (Appendix two, Figure 1). Aminoguanidine again appeared to be effective at inhibiting crosslinking (Figure 4.11 A) relative to the incubations that did not contain aminoguanidine (Figure 4.11 B). It has been found that addition of aminoguanidine to reaction mixtures containing glyoxal and protected lysine, results in the inhibition of some glyoxal-mediated crosslinks such as GOLD, but not others such as GOLA (57).

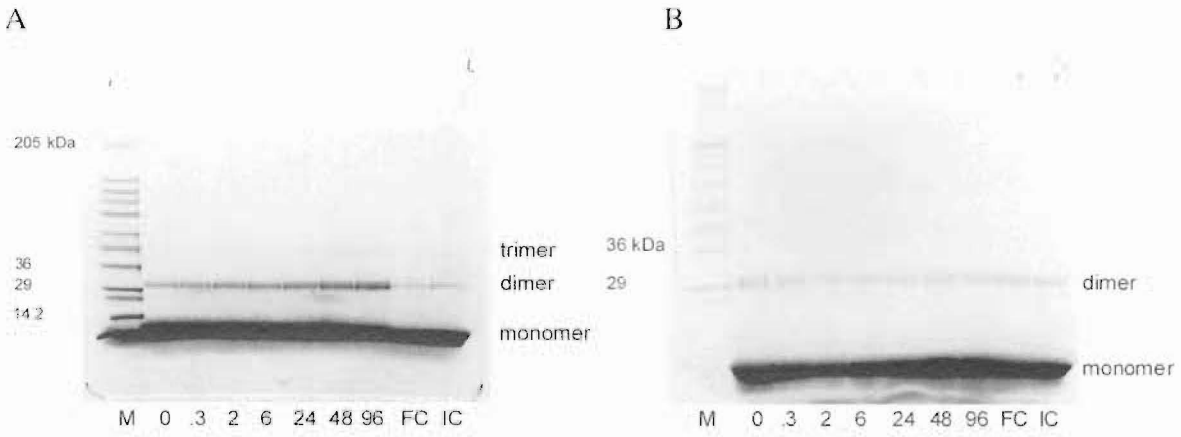


Figure 4.11: 8-16% SDS-PAGE gels of glyoxal incubated with RNase A in the absence A, or presence B, of aminoguanidine. Abbreviations are as for Figure 4.10.

A loss in activity of RNase A incubated with diacetyl was observed (Table 4.1) (Appendix two, Figure 2). Diacetyl has been reported to be an arginine modifier (58,59). Some reaction has also been reported, where diacetyl can crosslink protein with a concomittant decrease in measurable lysine residues (60). If modification of arginine is predominant, these results suggest that loss of arginine does not dramatically affect catalysis, although some loss in activity is observed.

Aminoguanidine again appeared to maintain the activity of RNase A that had been incubated with diacetyl (Table 4.1). Indeed, formation of triazines has been reported on the reaction of diacetyl with aminoguanidine under mild conditions (61), rendering diacetyl unable to react with RNase A to inactivate it. Crosslinking of RNase A by diacetyl and inhibition by aminoguanidine is illustrated in Figures 4.12 A and 4.12 B, respectively.

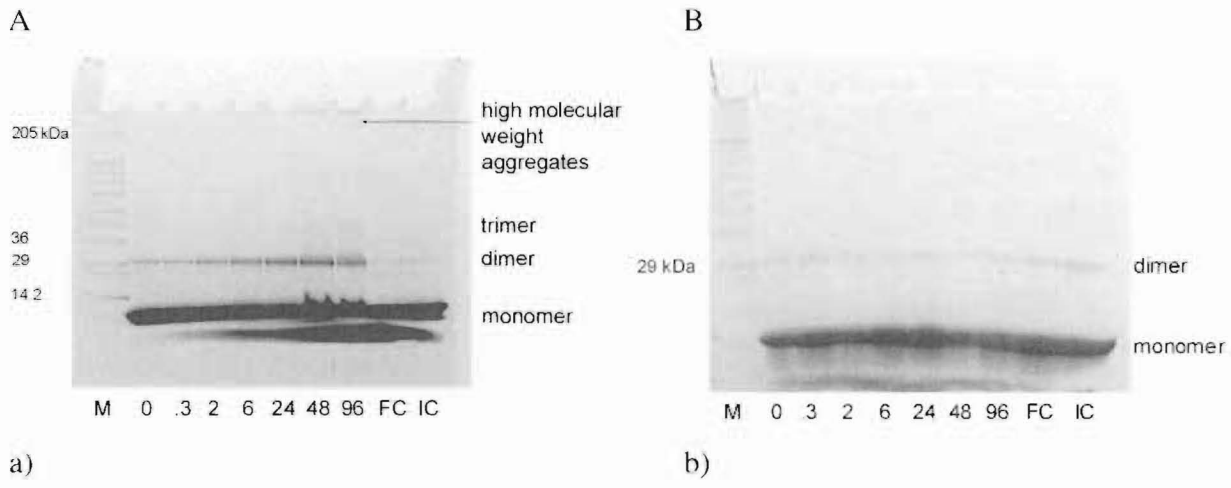


Figure 4.12: 8-16% SDS-PAGE gels of diacetyl incubated with RNase A in the absence A, or presence B, of aminoguanidine. Abbreviations are as for Figure 4.10.

4.4.2 DMPC

Another reported Maillard reaction inhibitor, DMPC, was included to assess its efficiency at preserving the activity of RNase A and inhibiting protein crosslinking. Activity of RNase A, incubated with both methylglyoxal and DMPC was found to decrease over time relative to the controls (Figure 4.13). This loss in activity paralleled activity of RNase A incubations that did not contain DMPC.

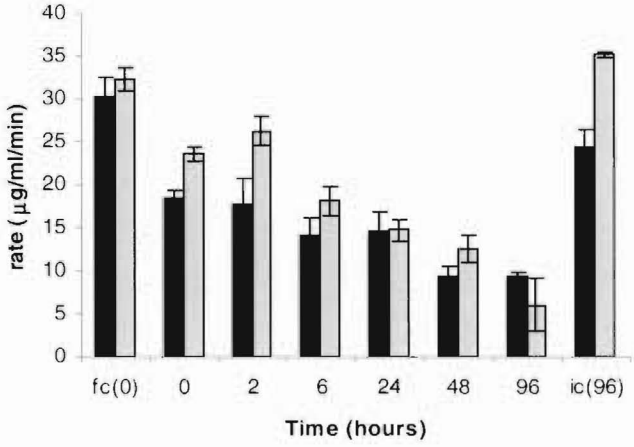


Figure 4.13: RNase A activity over 96 hours following incubation with methylglyoxal in the presence (■), or absence ( ) of 3,5-dimethylpyrazole-1-carboxamidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate reading. Abbreviations are as for Figure 4.10.

Another reported function of DMPC is that it acts as a guanidylating agent, converting a primary amine to a guanidino functionality (62-64). Interestingly, it has been reported that guanidination of 9 RNase A lysine residues by this method does not render the protein catalytically inactive, but modification of the final lysine (Lys41) does (64). This may explain the decrease in the activity of the incubated control (IC96) in Figure 4.13 that had been incubated in the presence of DMPC relative to the incubated control that did not contain DMPC. Guanidination may have resulted in the modification of critical lysines residues within RNase A. However, an optimal pH of 9.5 is required for guanidination (65), which was not reached in this work, although some enzymatic inactivation has been observed at pH 7.4 (66). Thus, a pH of 7, which the incubations were prepared at, may still favour a small amount of guanidination.

Figure 4.14 reflects the extent of crosslinking observed for methylglyoxal on addition of DMPC.

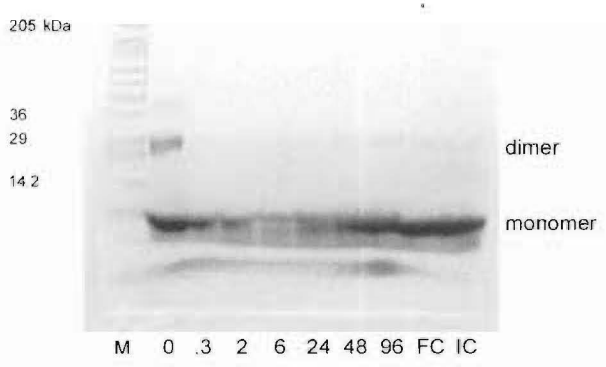


Figure 4.14: 8-16% SDS-PAGE gel of RNase A incubated with methylglyoxal in the presence of DMPC. Abbreviations are as for Figure 4.10.

These results with DMPC are in contrast to aminoguanidine, where crosslinking was inhibited and the function of RNase A was maintained. Although crosslinking of RNase by methylglyoxal is inhibited by DMPC (Figure 4.14), the function of RNase A has decreased steadily over time in the presence of this inhibitor. This will be further discussed in section 4.5.

On incubation of protein with glyoxal in the presence of DMPC, the inhibitor appeared to be more effective at retaining RNase A activity than methylglyoxal

(Table 4.2) (Appendix two, Figure 3). Crosslinking of RNase A by glyoxal was inhibited by DMPC (Appendix two, Figure 4).

Dicarbonyl	DMPC		No inhibitor	
	Crosslinking [a]	Rate of reaction (μg RNA/mL/min) [b]	Crosslinking	Rate of reaction (μg RNA/mL/min)
Methylglyoxal	-	9.4 ± 3.1	+++	3.8 ± 1.6
Glyoxal	-	25.4 ± 0.9	+	17.9 ± 0.7
Diacetyl	-	23.6 ± 0.5	+	22.1 ± 0.9
No Dicarbonyl	-	27.1 ± 1.3	-	28.8 ±1.1

Table 4.2: Comparison of RNase A crosslinking with catalytic activity. [a, b] error and replicates are exactly as outlined in Table 4.1 legend.

RNase A activity was impaired on incubation with diacetyl, and, inclusion of DMPC did not appear to have a protective effect with regard to activity of the protein (Table 4.2) (Appendix two, Figure 5). Inhibition of crosslinking of RNase A was observed in incubations containing diacetyl and DMPC (Table 4.2) (Appendix two, Figure 6).

The differences in RNase A activities in the presence or absence of DMPC suggest that modification of RNase A by methylglyoxal or diacetyl to protein-bound amine is favoured over reaction with DMPC, thus activity is not maintained. In the case of glyoxal-treated RNase A, maintenance may be due to glyoxal reacting preferentially with DMPC over protein-bound amine.

4.5 General discussion and conclusions

The work in this chapter probed the relationship of crosslinking to function of a model protein. Maillard inhibitors were included to further understand this relationship. Both aminoguanidine and DMPC inhibited the protein crosslinking reaction by all three α-dicarbonyl compounds with RNase A. In the presence of aminoguanidine, enzyme activity was retained, suggesting that this compound does indeed afford some protection against loss of protein function. However, in the presence of DMPC, despite the inhibition of protein crosslinking on incubation with RNase A and

methylglyoxal, the activity of the protein was lost. Thus an ability to inhibit protein crosslinking by the Maillard reaction does not necessarily guarantee that the damaging effects of protein glycation on protein function have been circumvented. Figure 4.15 summarises these findings and suggests a possible explanation for the difference in behaviour between the two inhibitors. These results support the view that aminoguanidine is effective at inhibiting crosslinking and preventing inactivation of the protein, since it reacts with α-dicarbonyls at a much faster rate than they are able to react with proteins. This does not always seem to be the case with DMPC, which does not react with methylglyoxal quickly enough to prevent it from reacting with the protein. While it does render the intermediate incapable of protein crosslinking, the damage, in terms of loss of protein function, is already done (Figure 4.15). Aminoguanidine may be more effective due to its ability to trap α-dicarbonyls *via* the hydrazine group, which is a better nucleophile than the guanidinium functionality of DMPC.

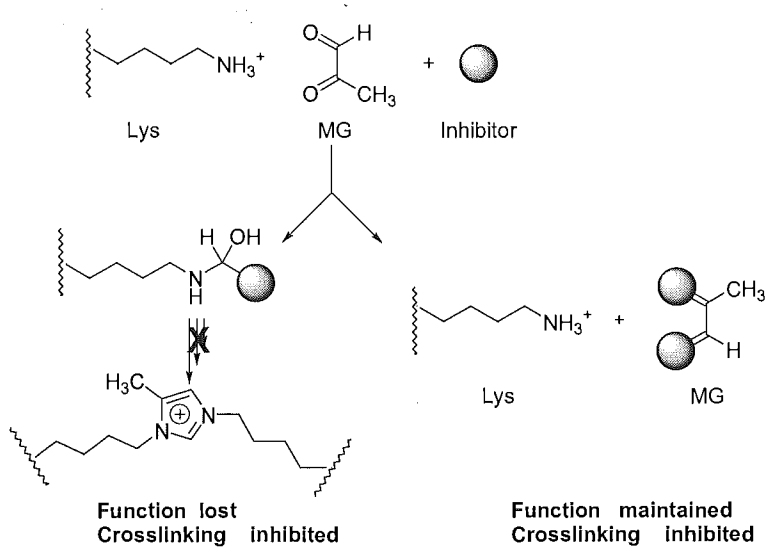


Figure 4.15: Possible reaction pathways for lysine modification by methylglyoxal reaction with lysine *in vitro*

It is tempting to speculate on the mechanism of inactivation of RNase A by methylglyoxal. Inactivation may be arising from reaction with lysine *via* steps outlined in Figure 4.15 or by a similar mechanism. Modification of lysine has resulted in a loss in function of RNase A (12). Recent work has confirmed that lysines 41 (critical for catalysis), 1, 7, and 37 are modified in RNase A on reaction with glucose (67). Another possibility is that methylglyoxal may react with arginine residues

(Figure 4.16), such that these modifications may ultimately result in the modification of enzymatic activity. A small proportion of Lys41 modification by glyoxal was also noted by these workers, and may explain the inactivation observed in this work when incubating glyoxal with RNase A (67).

Interestingly, Takahashi suggested that as RNase A could be inactivated by phenylglyoxal, a selective arginine modifier under certain conditions, the loss in function in the enzyme was due to modification of Arg39 (14). One must consider the possibly of arginine-lysine crosslinks (Figure 4.16) which could also potentially modify catalytically important lysine and arginine residues.

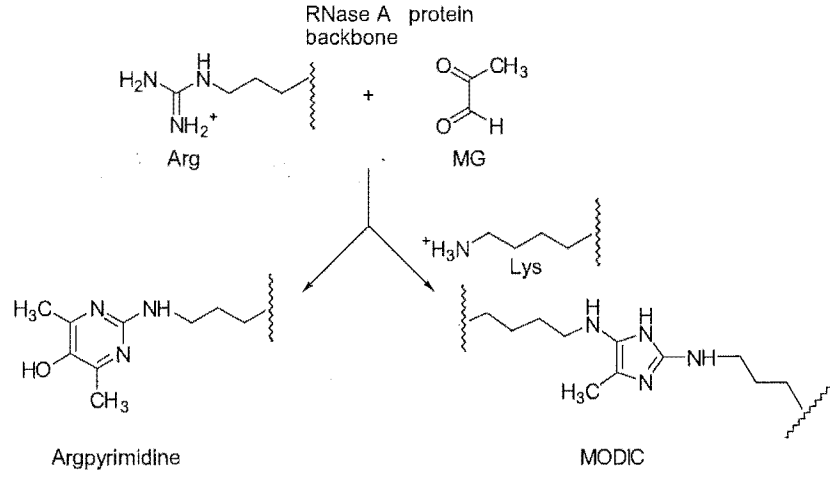


Figure 4.16: Possible reaction pathways of arginine on reaction with methylglyoxal in the presence of a Maillard reaction inhibitor *in vitro* (68,69).

These results demonstrate that preventing protein crosslinking does not necessarily preserve enzyme activity. These results cast doubt on the likely efficacy of some purported anti-ageing compounds *in vivo,* and emphasise the danger of generalising the results from simple model studies. Based on these results, the efficacy of Maillard inhibitors as anti-ageing therapeutics is likely to depend on many complex factors, the outcome of which will depend on not only the individual carbonyl moiety in question, but also the specific protein affected.

4.6 References

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Chapter Five

In search of amadoriase - developing kinetic assays to characterise protein deglycating enzymes

5.1 Introduction

The previous chapter studied the effect of glycation on protein function. An intimate knowledge of the effects of the Maillard reaction on proteins *in vitro* will shed more light on the detailed *in vivo* processes of diseases, such as diabetes and cataractogenesis, which are thought to involve the Maillard reaction. Better strategies can be designed to reverse or attenuate this chemistry as more becomes known about the physiological effects of glycation.

Advances in gene technology have meant that exogenous genes can be expressed in human systems for medical purposes (1). The approach, termed gene therapy, has broadened the scope of alternative approaches to intervention in the Maillard reaction to include enzymes that can reverse the Maillard reaction. These enzymes, termed deglycating enzymes, can reverse the early stages of the reaction by cleaving the Amadori product, generally liberating the original carbonyl compound and amine. One promising avenue of research involves the fructosyl amine-oxygen oxidoreductase deglycating enzymes that are so far confined to bacteria and fungi. These enzymes have the capacity to cleave the Amadori product that forms from the reaction between low molecular weight amines and sugars (Figure 5.1) (2-6). These

enzymes are commonly referred to as amadoriases (6), due the fact that the Amadori product is the substrate that is recognized by the enzyme.

In more recent years, it has been discovered that humans and mice possess deglycating enzymes, the ATP-dependent fructosamine kinases (7-9) (Figure 5.1). These also act on the Amadori product, but differ in mode of action from the amadoriases, in that they require ATP for their function.

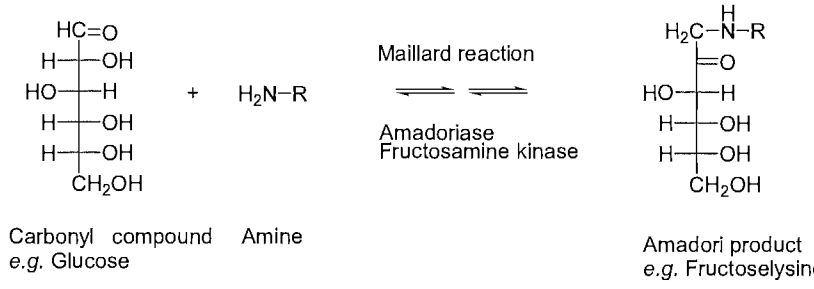


Figure 5.1: Enzymatic reversal of early stages of the Maillard reaction *via* either amadoriase or fructosamine kinase. Abbreviation: R, remainder of amine compound.

The fructosamine kinases act to phosphorylate the Amadori product formed on protein, which subsequently decomposes to regenerate an unmodified lysine residue, inorganic phosphate and 3-deoxyglucosone (Figure 5.2). Some enzymes have been shown to phosphorylate the third (7), or sixth carbon of fructoselysine, the Amadori product formed on reaction of glucose with lysine (Figure 5.2) (9). The spontaneous degradation of fructosamine-3-phosphate has been attributed to the fact that the phosphate is in close proximity to the carbonyl and thus will be susceptible to elimination (7,8). Studies where a fructoselysine 6-kinase was isolated also resulted in the discovery of a novel deglycase, fructoselysine-6-phosphate deglycase with a molecular weight of 405 kDa, which converted fructoselysine-6-phosphate to glucose-6-phosphate and lysine (9).

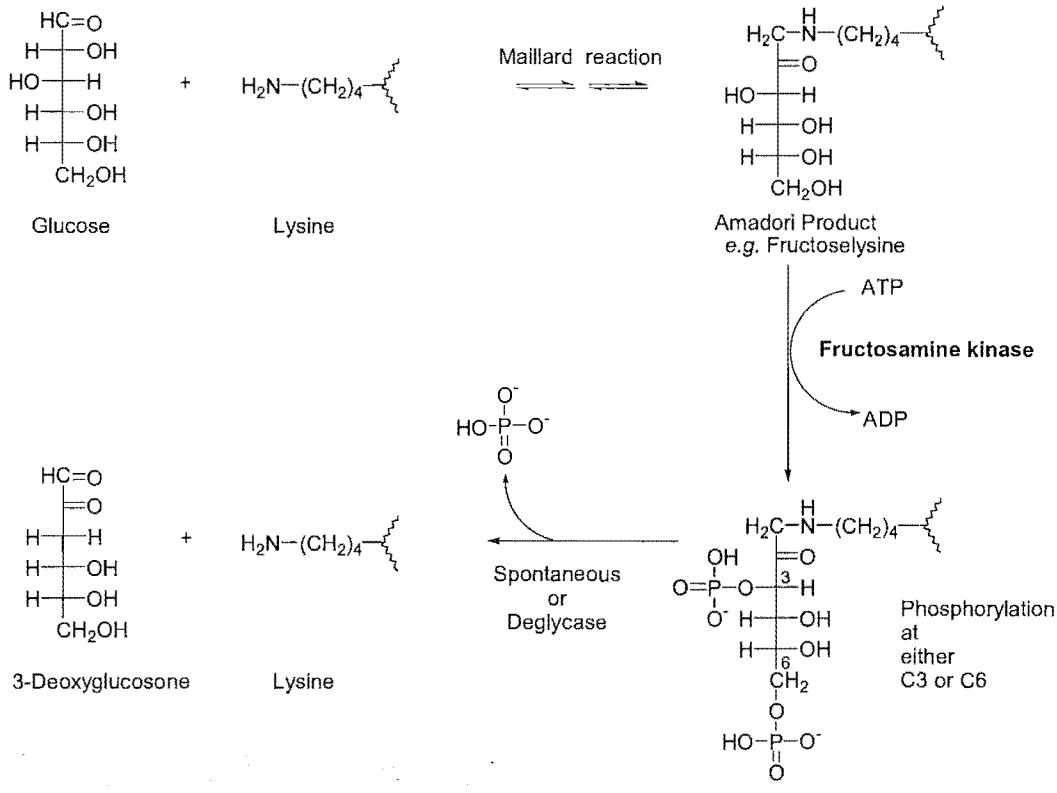


Figure 5.2: The deglycation pathway involving fructosamine kinase. Fructosamine kinase can either act at the 3C position (fructosamine-3-kinase) (7,8), or at the 6C position (fructoselysine 6-kinase) (9).

Chapter one highlighted the importance of the Maillard reaction in the progression of diseases such as diabetes (10). Thus, intervention in the Maillard reaction is paramount and a number of routes have already been explored (Chapter one). Ultimately, it is hoped that both types of deglycating enzymes, the amadoriases and the fructosamine kinases, could be expressed in patients with Maillard-related diseases, *via* gene therapy, to provide a means for alleviating many of the physiological complications encountered by diabetics, such as cataracts and neuropathy. A considerable barrier to this approach is that the amadoriases cannot act on glycated protein, the physiologically relevant substrate (4-6). This later point will discussed in more detail later in this chapter.

Deglycating enzymes have also been considered for use in DNA recombinant technology, for stripping off glycation adducts from recombinant proteins to generate safe, stable proteins (11). A final potential use for these enzymes is for diagnostics. A selection of amadoriase enzymes isolated to date has been cultured, with a view to

developing an assay for the quantification of the extent of hemoglobin glycation at the *N*-terminus in diabetic patients (12-14). This is proposed to act as an indicator of the extent of Maillard reaction occurring in patients. *In vitro* protein evolution has aided the development of this as a diagnostic tool, by generating a more thermostable and specific enzyme for the quantification procedure (15).

The remainder of this chapter will focus on the fructosyl amine-oxygen oxidoreductase, amadoriase I (6), which has not been reported to use glycated protein as a substrate. It was hypothesised that this inability to cleave Amadori products formed on protein is due to the fact that previous studies have used heavily glycated protein substrates (4-6). It may be that the enzyme can use a mildly glycated protein substrate, but beyond a certain threshold of glycation, is inactive.

A key bottleneck in the characterisation of any enzyme is the development of accurate, time resolved assays (16). This is true for the amadoriases. Therefore, this chapter details the development of two assays: a time-resolved assay for initial purification and kinetic characterisation purposes, using fructosyl propylamine as a substrate; and a novel method to assess amadoriase I activity using glycated protein as a substrate, based on the RNase A activity detailed in Chapter four.

With assay methods in hand, site-directed mutagenesis studies of amadoriase I was undertaken. The amino acids involved in the catalytic mechanism of amadoriase I are, to date, undefined. Previous studies proposed three possible modes of catalysis (17), consistent with those reported for enzymes that share homology (18,19). Thus, two mutants were generated to further probe the catalytic mechanism of this enzyme in order to contribute to the current understanding of the enzyme. The mutations were performed at sites that were thought to be critical for catalysis. Using the time-resolved method, the activity of two mutants of amadoriase I, created in collaboration with Andrea Uhlmann, Technische Universität München, Germany (His357 to Asn) and Stephan Hegge, Universität Hannover, Medizinische Hochschule Hannover, Germany (S370A), was successfully measured (Appendix four).

Finally, the second assay method established in this chapter was used to the search for deglycating enzymes in other organisms that could act on glycated protein. Attempts

to isolate a deglycating enzyme from *Pseudomonas fluorescens,* using this assay approach are outlined in Appendix five. This work was undertaken collaboratively with Sarah Day, an M.Sc. student at the University of Canterbury.

5.2 Amadoriase enzymes isolated to date

A summary of all of the amadoriase enzymes isolated to date, and their reported properties, are outlined in Table 5.1. Many of these enzymes have been isolated from organisms that have been cultured on low molecular weight Amadori product substrates, such as fructosyl aminocaproic acid, *i.e.* relatively small Amadori products with respect to a glycated protein substrate (Figure 5.3). As a result, these enzymes do not recognize larger substrates (2,20). Those enzymes that have been shown to exhibit an ability to deglycate a number of fructosyl amino acids do not recognise glycated protein (2,3). Some enzymes show the capacity to deglycate proteins, but only after protease treatment of the protein (12,14). A recent publication reports the ability of an enzyme that can act on glycated dipeptide (21).

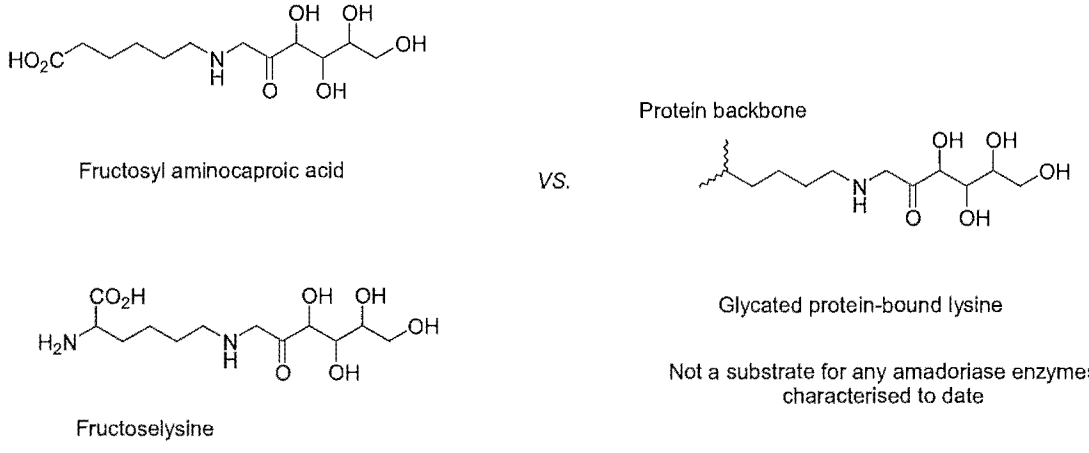


Figure 5.3: A selection of substrates employed in amadoriase studies to date. Those on the left are recognised by some amadoriases. Glycated protein however, is not recognized. The structure on the right is a reaction product of the reaction of glucose with protein-bound lysine (22), but has not been characterised in experiments where glycated protein has been used as a substrate (3,4).

Source	MW (Da) [a]	Substrate [b]	Prosthetic group	Products [c]
Corynebacterium (1)	44,000*	Fructosyl glycine	FAD	Glucosone, glycine, H_2O_2
Aspergillus (2)	41,500*	Fructosyl ε-aminocaproate	FAD	Glucosone, glycine, H_2O_2
Penicillium (18)	NR	Difructosyl lysine	NR	NR
Klebsiella, Corynebacterium	NR	Fructosyl valine	NR	Glucosone, butylamine, H_2O_2
Fusarium, Acremonium		Butylamino deoxyfructose		
Debaromyces (11)				
Penicillium (12)	39,000	Fructosyl valine	FAD	valine, glucosone, H_2O_2
Pseudomonas (3) (4)	106,000	Fructosyl ε-aminocaproate	NR	6-oxohexanoic acid, fructosamine
Aspergillus terreus (13)	53,000*	Frutosyl lysine	FAD	lysine, glucosone, H_2O_2
Aspergillus fumigatus (5,21)	51,000	Fructosyl adamantanamine	FAD	glucosone, propylamine, H_2O_2
Aspergillus fumigatus (5)	49,000	Fructosyl adamantanamine	FAD	glucosone, propylamine, H_2O_2
Aspergillus nidulans (22)	48,000	Fructosyl propylamine	FAD	Glucosone, propylamine, H_2O_2
Achaetomiella virescens (19)	50,000	N^{α} Fructosyl valyl-histidine	Flavin	glucosone, valyl-histidine, H_2O_2

Table 5.1: Deglycating enzymes (that do not require ATP) isolated thus far. [a] Molecular weight of monomer as judged by size exclusion chromatography. [b] Substrate used as the sole carbon source in experiments to isolate deglycating enzymes. [c] Products that arise on catalysis of model substrates used to characterise the deglycating enzyme. *N.B.* the substrate used for isolation of the deglycating enzyme (column 2) can differ to that used to further characterize the enzyme. * denotes that the protein exists as a dimer, otherwise, protein exists as a monomer. NR, Not reported in the source publication.

Most fructosyl amine-oxygen oxidoreductases characterised cleave the Amadori product at the ketoamine bond (Figure 5.4 A). One enzyme, however, isolated from *Pseudomonas*, cleaves at the alkylamine bond (Figure 5.4 B) (4,5). Interestingly, these enzymes were found to act on ribated protein (protein incubated with ribose), but not protein treated with glucose (4).

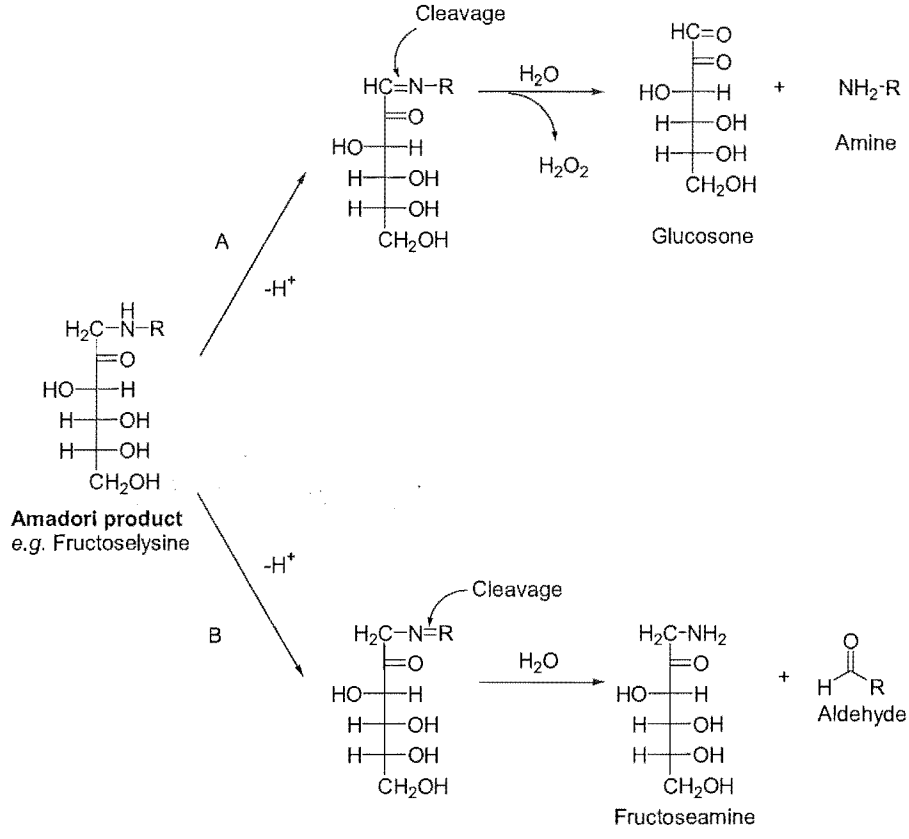


Figure 5.4: Deglycation of an Amadori product *via* enzymes that catalyse A, ketoamine bond cleavage or B, alkylamine bond cleavage. R denotes the remaining portion of lysine. After Gerhardinger *et al.* (4).

In recent years, two amadoriase isozymes, amadoriase I and II have been the subject of intense study, and as a result, more is becoming known about their mechanisms and the amino acid residues required for their function (17,23,25). Our understanding of amadoriase regulation and the role it plays in the organism it was originally isolated from has been extended by gene regulation studies. These studies show that a deglycating enzyme isolated from *Aspergillus nidulans* could not be induced solely by fructosylamines, and the *veA* gene was a co-requirement for induction of amadoriase (24).

5.3 The amadoriase I and II isozymes

Amadoriase I and II were first isolated from *Aspergillus fumigatus* that had been grown on a highly sterically inhibited Amadori product, in order to mimic the structure of a glycated protein. These enzymes were found to cleave at the ketoamine bond to produce glucosone and free amine from the Amadori product, along with hydrogen peroxide (Figure 5.4) (6). The expression of these amadoriases, was found to be induced by Amadori compounds. Both enzymes were cloned and expressed in *E. coli* (23,26). Amadoriase I was the deglycating enzyme that was chosen to test protein deglycating ability and the two new assay methods. The plasmid bearing the gene that encoded for this enzyme was kindly provided by Prof. Vincent Monnier (Case Western University, Cleveland, Ohio).

5.3.1 The proposed catalytic mechanisms of amadoriase I

Spectral studies showed that amadoriase I contained an FAD prosthetic group (6). Mutation of Cys342 of amadoriase I suggested that this was the residue to which the FAD prosthetic group was covalently attached (23). Later work showed that amadoriase I bound a cyclic β isomer of the substrate, fructosyl propylamine (17). Also outlined in that work was a pre-steady state analysis of the amadoriase I mechanism, in which a complex absorption spectrum for an intermediate was resolved (17).

The proposed kinetic mechanism of amadoriase I is thought to proceed *via* an ordered ternary complex (Figure 5.5). This ternary complex contains three substituents: enzyme, reduced flavin and product, and is represented in Figure 5.5 as $E{\sim}FL_{red}{\sim}P$. The term ordered refers to the fact that substrates must bind to the enzyme in a particular sequence. Adherence to this sequence is crucial to the enzyme's ability to catalyse the reaction. For amadoriase I, the binding of the substrate fructosyl propylamine (k_1) precedes the binding of oxygen (k_6), the second substrate (Figure 5.5).

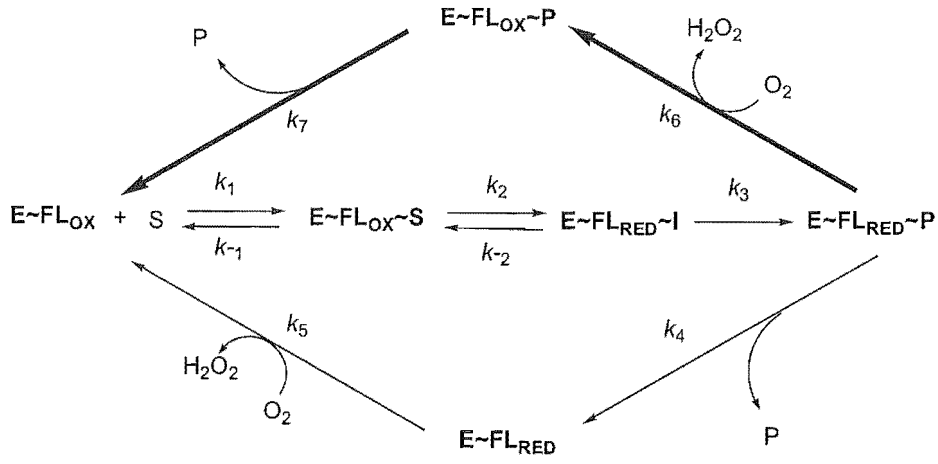


Figure 5.5: The proposed kinetic mechanism for the catalytic cycle of amadoriase I. Thicker arrows represent the actual pathway following turnover. E, enzyme; FL_{ox}, oxidized flavin; FL_{red}, reduced flavin; S, substrate; I, intermediate; P, product. From Wu *et al.* (17).

The catalytic mechanism of this family of enzymes of which amadoriase I is a member, the FAD dependent oxidoreductases that act on secondary or tertiary amino acids, is open to debate. Another member of this family, monomeric sarcosine oxidase from Bacillus *sp.* shares 22% sequence homology with amadoriase I (23). Inspection of BLAST sequences shows that this is the protein that is most similar to amadoriase I with a published X-ray structure (18). Trickey *et al.* report that the structure of the monomeric sarcosine oxidase may provide a model for other members of this family (18). Inspection of the proposed catalytic mechanisms for this family of enzymes suggests three possibilities for the substrate dehydrogenation, a critical step in catalysis: a polar nucleophilic mechanism, a carbanion mechanism and a direct hydride transfer mechanism (18,27-29). All of these may operate in amadoriase I (Figure 5.6) (17), and will each be discussed in turn.

Before the nucleophilic mechanism can take place, it is thought that ring-opening of fructosyl propylamine must occur (Figure 5.6A **1**). Nucleophilic attack of the substrate amine at the C4 of the covalently bound flavin then proceeds (Figure 5.6A **2**). Concomitantly, a proton is abstracted from an active site water or amino acid residue by the flavin (Figure 5.6A **2**). The substrate is deprotonated at the α-carbon by the same active site residue, with subsequent breaking of the covalent bond between the flavin and the substrate (Figure 5.6A **3**), and the generation of an

intermediate (Figure 5.6A **4**) which is oxidised to form the final products of the reaction. His 269 has been proposed to be the active site base in monomeric sarcosine oxidase that is involved in this mechanism (18). However, mutational studies have shown that this residue is critical for optimizing the orientation of bound substrate with respect to electron transfer to the flavin, and attention has been shifted to a tyrosine residue situated close to both the flavin and His 269 (30).

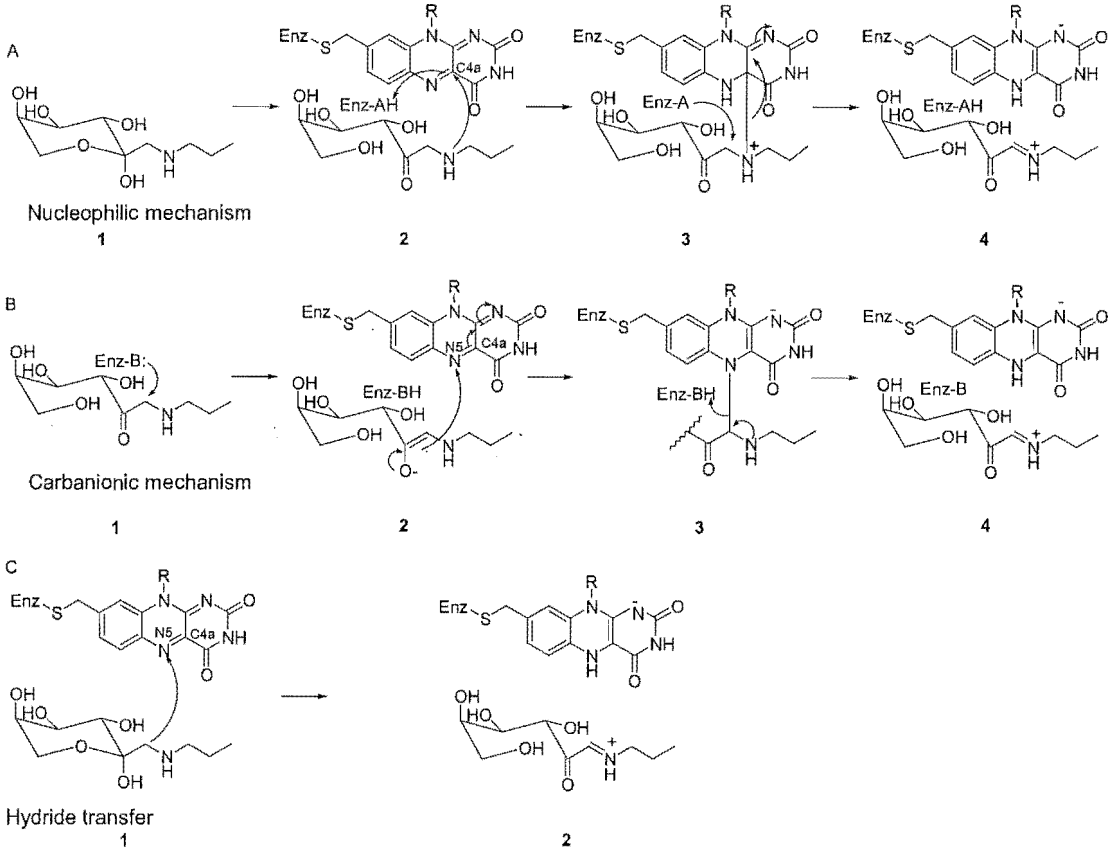


Figure 5.6: Three proposed catalytic mechanisms of amadoriase I. From Wu *et al.* 2001 (17). Abbreviations: Enz-AH, protonated active site amino acid residue A; Enz-A, deprotonated active site amino acid residue A; Enz-BH, protonated active site amino acid residue B; Enz-A, deprotonated active site amino acid residue B.

An active site base is also a prerequisite for another catalytic mechanism, the carbanion mechanism (Figure 5.6 B). Again, ring opening is thought to be required for catalysis. An active site base then deprotonates the substrate at the α-carbon of the substrate which then attacks the N5 of the flavin ring (Figure 5.6 B **1** and **2**) (31). After this, the active site base donates a proton to the substrate and the covalent bond to N5 is cleaved (Figure 5.6 B **3**) (17,29). However, Wu *et al.* note that carbanionic

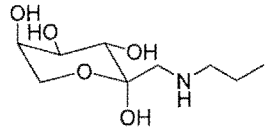
intermediates are generally unstable and the substrate would not offer any inherent stability (17).

The final mechanism to be detailed here is the direct hydride transfer (Figure 5.6 C). This has been suggested for *D*-amino acid oxidase, a member of the FAD dependent oxidoreductases, where the substrate is thought to donate a proton directly to N5 of the flavin (Figure 5.6 C **1**) (19,32).

However, none of the intermediates in the proposed catalytic schemes above have been identified (17).

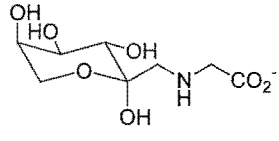
5.3.2 The proposed catalytic mechanisms of amadoriase II

The catalytic mechanism of amadoriase II has not been characterised to the extent that amadoriase I has. However, a substrate specificity study has shown that amadoriase II favours binding of a carboxylate group over an uncharged methyl group (Figure 5.7) (25). Double mutation of Arg 112 and Arg 114 of amadoriase II to Glu (as are found in amadoriase I) suggest that these residues are critical for anionic substrate binding but not catalysis (25).



Amadoriase I substrate

Fructosyl propylamine



Amadoriase II substrate

Glycine Amadori product

Figure 5.7: Preferred substrates for amadoriase I and II respectively.

5.3.3 Can amadoriase I use glycated protein as a substrate?

To date, attention has focused primarily on the action of amadoriase I on low molecular weight substrates. Thus, the research detailed below sought to examine whether amadoriase I could catalyse the deglycation of a mildly glycated protein substrate.

Amadoriase I has been shown to be inactive toward some glycated protein substrates derived from hexoses and pentoses, such as glucose and ribose. This study has used methylglyoxal to mildly glycate protein. This α-dicarbonyl was selected due to the current view that α-dicarbonyls, some formed on oxidation of glucose (Chapter one), are a more physiologically relevant species in terms of the Maillard reaction *in vivo* than hexoses (*e.g.* glucose) and pentoses (*e.g.* ribose) (33).

Methylglyoxal allows for modification of protein in a short time, minimising possible break-down problems with the incubated protein. Also, information regarding the rate of protein glycation by methylglyoxal was known from lysine estimation described in Chapter two, and was used to gauge the extent of the Maillard reaction. Strictly, Amadori product formation on reaction of methylglyoxal with amine does not occur, but the reaction may yield a product similar in structure, which may be recognized by amadoriase I (Figure 5.8).

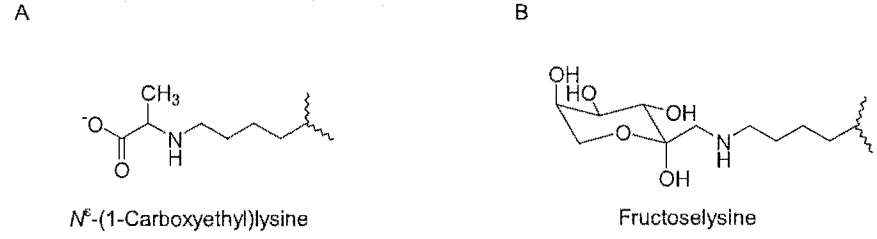


Figure 5.8: Reported structures on reaction products of A, methylglyoxal with protein (34) and B, the classical "Amadori product" fructoselysine formed on reaction of glucose with protein (35,36).

5.3.4 Current methods employed to monitor the activity of amadoriases

Some previous methods to quantify the rate of amadoriase activity have measured the rate of oxygen consumption. These methods take advantage of the fact that all deglycating enzymes isolated to date are oxygen dependent (Figure 5.9) (2-6).

An alternative route to measure activity is by quantification of another end product of the amadoriase catalysis, glucosone (Figure 5.9) (6,26). However, this method may result in underestimation of rate, due to the reported ability of glucosone itself to participate in the Maillard reaction (37), and the fact that it will easily fragment under oxidative conditions due to its structure (38).

More recently, production of hydrogen peroxide by these enzymes has been used as a means for assaying amadoriase (Figure 5.9). Hydrogen peroxide acts as a substrate for peroxidase and, in the presence of a second chromophoric substrate, the oxidation of the chromagen can allow for the measurement of amadoriase activity. Chromophoric and fluorogenic substrates have varied, and include quinone dye (26), ABTS (Figure 5.9) (17), Amplex® red (10-acetyl-3,7-dihydroxyphenoxazine) (24,25), 4-aminoantipyrine (15) and 4-aminophenazone (12). Determining activity by this coupled assay circumvents problems that arise with measurement of rate by the glucosone assay discussed above. This assay also provides a simple and reproducible means for quantifying amadoriase I activity, without the need for any specialized equipment, such as those needed for studies involving measuring oxygen consumption.

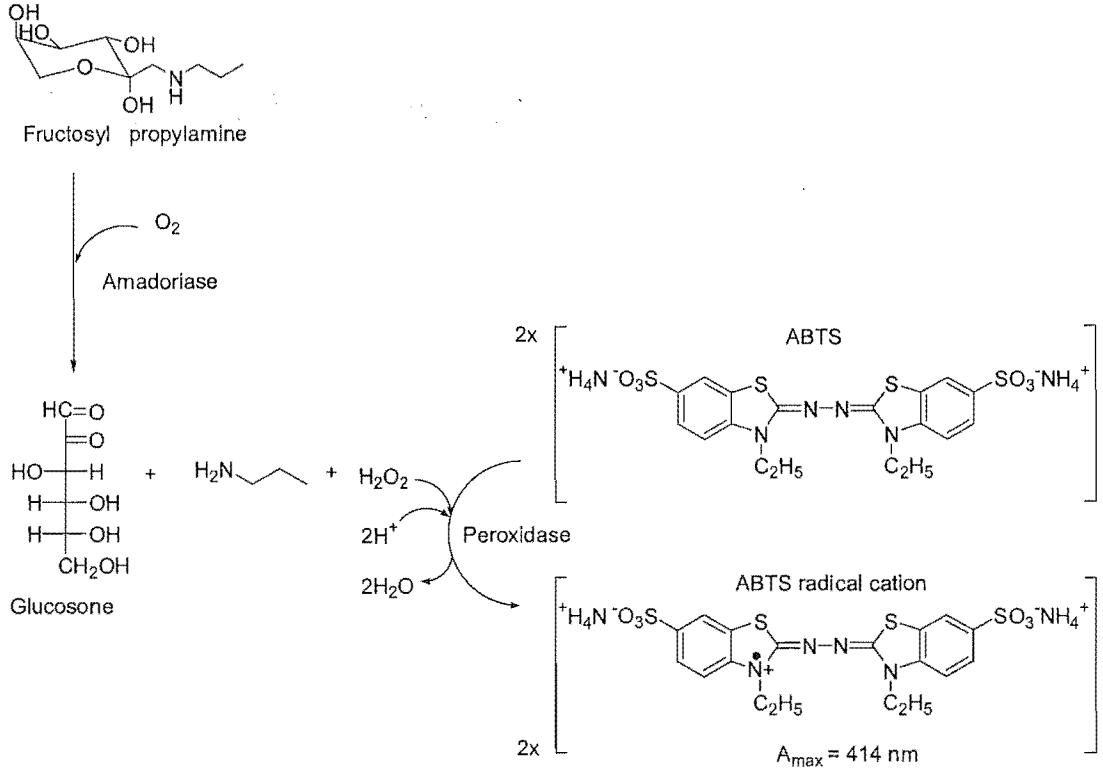


Figure 5.9: Coupled assay employed for the measurement of amadoriase I initial rate. After Childs and Bardsley (40). Abbreviations, ABTS, [2,2'-azino-di(3-ethyl-benzthiazoline-6-sulphonic acid)]. Two molecules of ABTS react with one H_2O_2 giving two molecules of radical cation.

The work undertaken here used the coupled assay approach, where amadoriase I was coupled to peroxidase. This enabled the detection of H_2O_2 consumption by the latter, measured by oxidation of a chromagen to yield a well-defined visible absorption spectrum. The chromagen selected was ABTS, due to its commercial availability and non-toxicity. In the presence of peroxidases, ABTS reacts with H_2O_2, produced by amadoriase I, to give the radical cation form of ABTS which absorbs at 414 nm (Figure 5.9). The assay for amadoriase I using ABTS has been previously performed as a discontinuous microtitre plate-based procedure (17). No real time assay for amadoriase is currently available and experiments outlined sought to remedy this.

An existing assay was adapted to a time-resolved, spectrophotometer-based assay, which eliminates inaccuracies when measuring initial rate. An assumption that arises during discontinuous assays is that the reaction is linear for all of the time prior to the last measurement (39). It may be that the reaction is linear for only a portion of the time (Figure 5.10). The development of a continuous kinetic assay has allowed for the accurate determination of initial rates, and the detection of subtleties of the reaction, that may have been overlooked when using the previous methods.

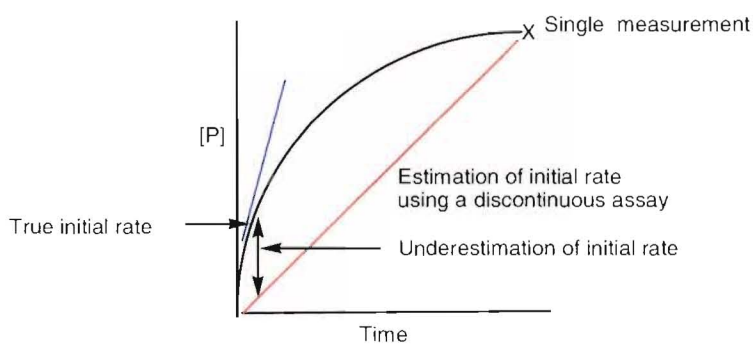


Figure 5.10: Discontinuous assays that measure one point after period of time can lead to underestimation of initial rate.

In addition to improving an existing assay, a new class of assay was developed. Until now, amadoriase I activity using glycated protein as a substrate, has been only tested by conventional methods such as measuring the consumption of oxygen (4), or the formation of glucosone (6). These methods measure substrate loss or product formation and only monitor the degree to which glycated protein substrate is turned over, not whether the protein has been restored to the same structural and functional

state as it was prior to glycation. Using the conventional assays, false positives may occur. For example, a portion of Amadori products may be removed from a protein by the amadoriase I enzyme, but the remaining modified residues may be critical for catalysis and thus the protein will still be inactive. This is important when considering active site lysine and arginine residues, which may not be accessible to amadoriase, but their modification can severely decrease activity.

These problems are circumvented using the novel method developed in this chapter, which involves, direct measurement of the activity of the glycated protein after treatment with amadoriase I. This method, employed in section 5.7, provides the critically important information from a therapeutic perspective: whether the protein has been restored to its native function after treatment with amadoriase I. The assay was derived from the RNase A assay outlined in Chapter four. Using this assay, it was shown that on incubation with methylglyoxal, RNase A lost activity (Chapter four). Thus, after treatment of amadoriase I, it was hypothesised that RNase A would have at least some activity restored as the protein was deglycated (Figure 5.11). A mildly glycated RNase A substrate was prepared, which showed some loss in activity, and this enabled the analysis of whether amadoriase I could at least partially restore function to glycated RNase A (Figure 5.11).

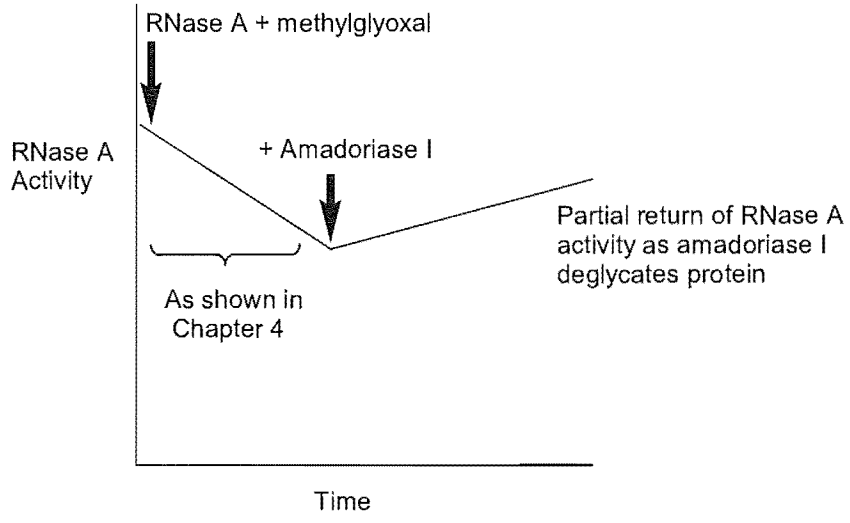


Figure 5.11: Hypothetical return of activity to RNase A is an indicator of the ability of amadoriase I to deglycate protein.

Before this deglycating capacity could be assessed, amadoriase I had to be over-expressed, purified and kinetically characterised. To do this, the time-resolved assay was developed, which allowed for the accurate measurement of initial rate afforded by a continuous assay. Each of these steps is outlined in the following sections.

5.4 Over-expression and characterisation of amadoriase I

5.4.1. Transformation of E. coli *with the plasmid containing amadoriase I*

The pET vector, containing the amadoriase I gene (1.5 kb) ligated into the plasmid between the *Bam*HI and *Nde*I sites (Figure 5.12) (23), was kindly provided by Prof. Vincent Monnier.

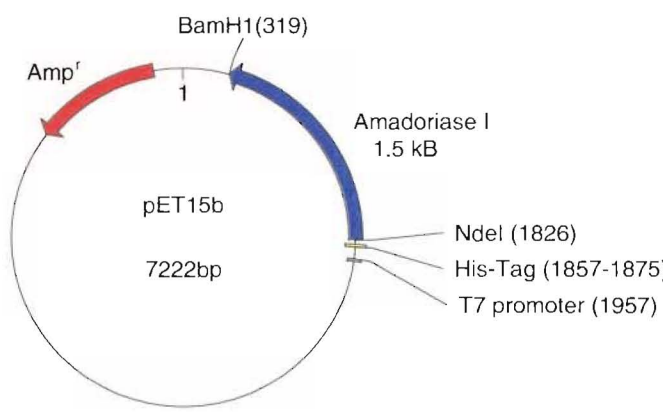


Figure 5.12: pET 15b vector map containing the amadoriase I insert. The 1.5 kb Amadoriase I insert lies between *Nde*I and *Bam*HI. Vector map was created using DNAMAN (Lynnon Corporation, Quebec, Canada). The numbers in brackets denote the base number.

This plasmid was used to transform *E. coli* BL21(DE3)pLysS for over-expression and purification of amadoriase I. This strain was selected due to its compatibility with the pET vector in terms of the expression system (41). It contains a T7 polymerase gene on the bacterial chromosome, whose expression is induced by isopropyl β-D-1-thiogalactopyranoside, IPTG (Figure 5.13). Once induced with IPTG, T7 RNA polymerase is produced, which in turn binds to the T7 promoter of the pET vector that

controls the expression of the target gene, in this case, amadoriase I (Figure 5.13). *E. coli* BL21(DE3)pLysS offers the advantage that it contains a regulatory plasmid, pLysS (Figure 5.13), which contains a gene that produces a small amount of T7 lysozyme to inactivate any basal levels of T7 RNA polymerase in the absence of IPTG (41). Thus, use of this strain allows for easy induction, tight control of amadoriase I expression (41), and rapid growth over a short period of time, allowing for efficient purification of amadoriase I.

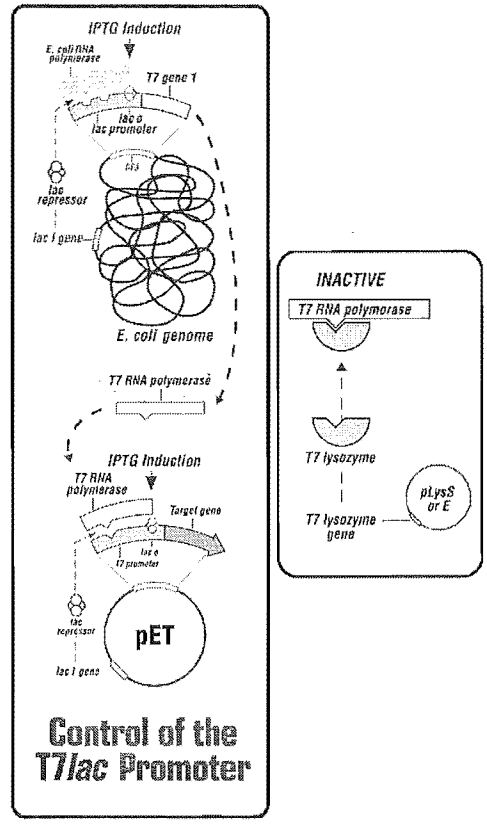


Figure 5.13: The mode of expression of amadoriase I from *E. coli* BL21(DE3)pLysS containing the pET15b containing the amadoriase I gene (41).

Antibiotic selection allowed for the strains that were successfully transformed by the pET vector (Cam^r Amp^r). The appropriate strain carrying the vector was cultured and the plasmid DNA purified. Initial problems were noted with the restriction digest pattern yielded on cleavage of the pET vector at *Bam*HI and *Nde*I sites when it had been prepared from *E. coli* BL21(DE3)pLysS (Figure 5.14 A).

The restriction digest showed the presence of a 0.5 kb fragment and multiple bands around the pET vector size. This was due to the interference of the regulatory

plasmid pLysS. pLysS contains a *Bam*HI site that will be recognised by the enzymes used in the restriction analysis experiments (42). However, after inspection of the sequence of the parent plasmid, pACYC184, and the T7 lysozyme sequence (inserted to create the pET vector), no other sites could be found that were cut by these enzymes, or their isoschizomers, that could account for the 0.5 kb band. The multiple bands were most likely due to the presence of the uncut pLysS vector that has a size of around 4.9 kb. These were at different positions in the gel relative to the cleaved vector due to the fact that the uncut DNA is in a supercoiled form which can affect its migration through the gel (43).

Transformation of *E. coli* XL1-Blue with the pET 15b vector circumvented this problem. *E. coli* XL1-Blue was selected due to the fact that it does not contain any other plasmids that affect the digest pattern and it is a common strain used for over-expression of proteins (44). Antibiotic selection allowed for the recovery of *E. coli* XL1-Blue strains successfully transformed by the pET vector (Tet^r, Amp^r). The expected 1.5 kb insert for amadoriase I was then yielded on digestion of the pET vector with *Bam*HI and *Nde*I (Figure 5.14B, lane 1). The apparent high molecular weight fragments shown in lane 2 of Figure 5.14B are indicative of uncut, supercoiled vector DNA, which have a differing mobility to the cut, linear DNA (43).

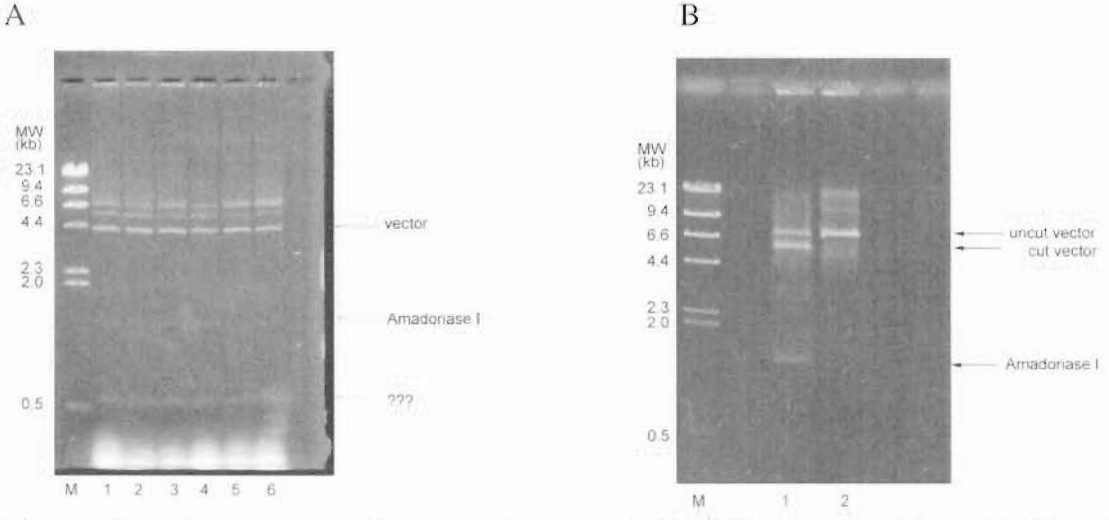


Figure 5.14: Agarose gels of restriction digests of pET15b prepared from A, *E. coli* BL21(DE3)pLysS: M, λ cut with *Hin*dIII; 1-6, DNA cut with *Nde*I and *Bam*HI B, *E. coli* XL1-Blue: M, λ cut with *Hin*dIII; 1, DNA cut with *Nde*I and *Bam*HI; 2, uncut DNA.

5.4.2 Amadoriase I purification procedure

Prior to purification, the substrate of amadoriase I, fructosyl propylamine, was synthesised by the method outlined in Takahashi *et al.* (6), and characterised by NMR, mass spectrometry and melting point analysis.

Purification of amadoriase I was undertaken as outlined by Wu *et al.* (23), with minor modifications. Purification was facilitated by the fact that the amadoriase I gene was expressed with a series of *N*-terminal repeats of His residues. Amadoriase I could therefore be purified by nickel chelating columns, where the His residues chelated with the nickel ions immobilized within the resin, to selectively retain the amadoriase I protein (45). The protein could then be eluted with imidazole, a competitor for the cation. This approach to purification is advantageous as it allows for purification to homogeneity in one step. A typical purification table from 1 L of culture is outlined in Table 5.2 and the SDS-PAGE gel that depicts the samples before and after elution through the column, is shown in Figure 5.15. After the column chromatography, only a 50 kDa band remained, which corresponded to the molecular weight of amadoriase I.

Step	*Total protein (mg)*[a]	*Total activity (Units)*[b,c]	*Specific Activity (Units/mg)*	*Yield* %	*Purification -fold*
Crude cell extract	109	18,400	169	100	1.0
His-Trap column	1.5	10,200	6,970	56	40

Table 5.2: A typical purification table for amadoriase I. [a] As determined by Bradford assay (46). [b] One unit of enzyme activity is defined as the amount of enzyme that produces 1 μM of H_2O_2 per min. [c] Activity measured using the ABTS assay with 0.49 mM fructosyl propylamine used as a substrate concentration of 10 x K_m reported by Wu *et al.* (23).

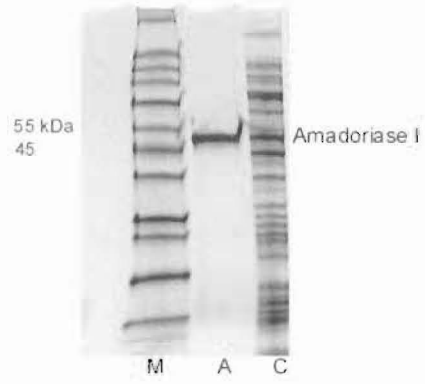


Figure 5.15: SDS-PAGE gel of amadoriase I purification. M, Marker; A, purified amadoriase I following elution from the Ni^{2+} charged column and subsequent dialysis; C, crude cell extract.

During early studies, a dramatic drop in activity of amadoriase I was noted during storage. Upon storage of the protein at -20°C for 2 days, the enzyme was found to retain 18% of its activity relative to when freshly prepared. After storage at 4°C or -80°C for 10 days, the enzyme retained 60% of activity relative to freshly prepared amadoriase I. This loss in activity was concomitant with a decrease in protein concentration as measured by the Bradford assay, suggesting that the protein was unstable and degraded over time. This confirms other reports of instability of amadoriases at 4 and -20°C (5). Further studies showed that after an initial drop in activity to 60% relative to fresh amadoriase I, the stored protein retained this level of activity for around 2 to 3 months. Considering the above results, a storage temperature -80°C was selected for amadoriase I.

5.5 Experiments to determine K_m and V_{max} of amadoriase I

The enzyme was further characterised by measuring its apparent kinetic parameters, K_m, V_{max} and k_{cat}, using the time-resolved coupled assay outlined above. Prior to data collection, experiments were undertaken to confirm that enzyme concentration was proportional to rate (Figure 5.16). Results for a typical experiment to determine K_m and V_{max} are depicted in Figure 5.17, along with comparison to existing literature values (Table 5.3). The substrate concentration range was between 6 K_m and 0.2 K_m of the literature value for fructosyl propylamine (23). This range was consistent with that stipulated by Cornish-Bowden for experiments to determine K_m and V_{max} (47).

The concentration of oxygen, the second substrate, dissolved in solution at 25°C was taken as 0.26 mM (48).

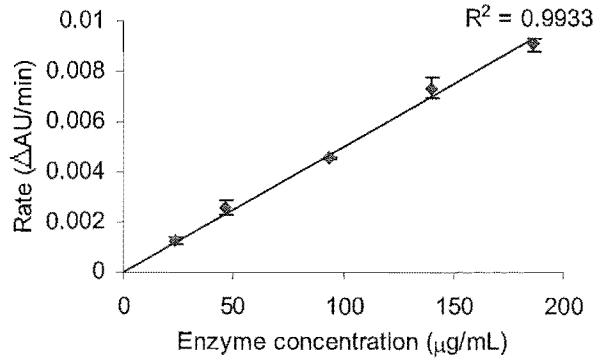


Figure 5.16: Enzyme concentration is proportional to rate. Rate is expressed as change in absorbance at 414 nm per min. Each point is an average of triplicate samples. Error is expressed as standard error of the mean for triplicate samples.

It appears that the data gained from these experiments fit Michaelis-Menten kinetics well (Figure 5.17 A, C, D). The results for each replicate experiment were found to be highly reproducible from day to day. The residual plot (Figure 5.17 B) shows random distribution of residual rate when plotted against concentration of substrate. Any ordering of the plot can signify a number of differing aspects of the data including that the data are not normally distributed (49). The residual plot for amadoriase I does not show any apparent ordering, confirming that the data fit the normal distribution.

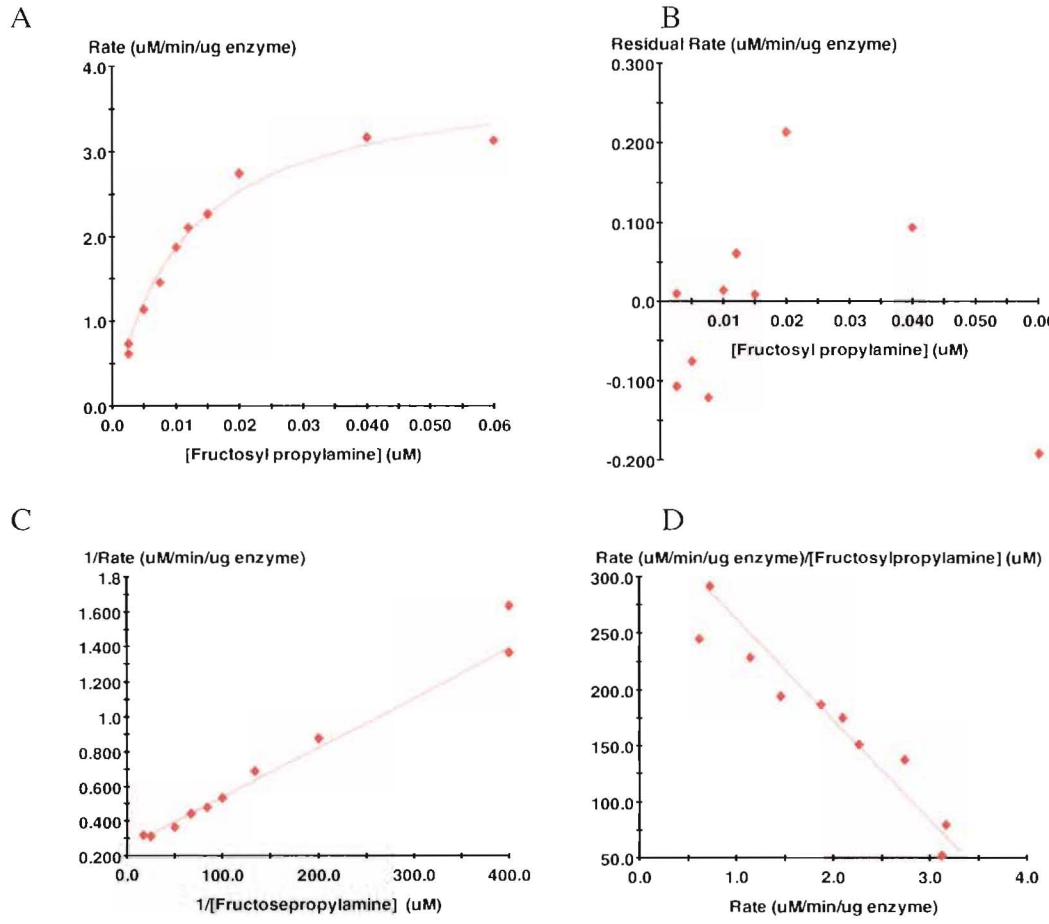


Figure 5.17: Michaelis-Menten kinetics of amadoriase I expressed as A, a direct Michaelis-Menten plot; B, a residual plot; C, a Lineweaver-Burk plot; D, an Eadie-Hofstee plot. R^2 of fit to Michaelis-Menten equation for A, B, D = 0.98, $P < 0.05$. Each data point is an average of three readings.

Assay system	K_m^{app} (mM)	k_{cat}^{app} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
Glucosone (26)	0.049	1.96	$1.31x10^5$
Amplex red (25) (H_2O_2 production)	0.023	5.8	$3.86x10^5$
This assay [a]	0.011 ± 0.0004	3.5 ± 0.3	$3.25x10^5 \pm 0.24x10^5$

Table 5.3: Apparent kinetic parameters of amadoriase I. [a] Values are an average of triplicate experiments, with duplicate readings of each point within the experiments.

An apparent K_m of 11 µM for fructosyl propylamine was calculated from these experiments. This shows that half of the maximal velocity of amadoriase I is reached at 11 µM fructosyl propylamine, thus amadoriase I has a high affinity for fructosyl

propylamine. The Michaelis constant reported here is a factor of 2 and 5 lower than those previously reported (Table 5.3). This is consistent with previous research under-estimating the true initial rate measured as detailed above (Figure 5.10).

An apparent k_{cat} of 3.5 sec^{-1} was also calculated from the data from these experiments. k_{cat} represents the maximum number of substrate molecules converted to products per active site per unit time, or the number of times the enzyme turns over per unit time (50), at a concentration of 0.26 mM O_2. k_{cat}/K_m is a measure of catalytic efficiency with catalytically "perfect" enzymes operating around 10^8-10^9 and those at 10^5, such as amadoriase, still considered very efficient at binding and turning over the substrate (50).

5.6 Substrate specificity of amadoriase I

To date, amadoriase I work involving substrate specificity has focused on the structure of the sugar residue that constitutes part of the substrate (17). Since, Amadoriase I shares 22% homology with monomeric sarcosine oxidase isolated from *Bacillus sp.* (section 5.3.1) (23), the possibility of amadoriase I having a capacity to turn over any of the substrates of sarcosine oxidase was tested. A number of these substrates were tested and the results are summarised in Table 5.4. Each sample was trialled at 10 times the quoted literature K_m or K_d in order to ensure that the substrate tested was not rate-limiting.

Substrate	Literature K_m (mM) [a, b]	[Assay] (mM)	Substrate for Amadoriase I?
N-methyl-*DL*-alanine	3.3 ± 0.2 (52) 6.8 (53)	27.2 (4xK_m of ref 52)	No
Sarcosine	4.5 ± 0.1 (52)	45 (10xK_m of ref 51)	No
Butyrate	**118 (51)**	**118 (1xK_d ref 53)** [b]	No
L-Proline	130 ± 10 (51)	260 (2xK_m of ref 51)	No
N-methyl-*DL*-valine	173 (53)	173 (1xK_m of ref 52)	No
Glycine	**359 (51)**	**359 (1xK_d of ref 53)**	No

Table 5.4: Substrate specificity of amadoriase I tested using sarcosine oxidase substrates. [a] Bold type is K_d data rather than K_m data. [b] K_d in this case is defined as the complex dissociation constant, with the complex being the enzyme with substrate bound (53).

Amadoriase I did not appear to turn over any of the sarcosine oxidase substrates tested (Table 5.4). This has also been reported for the enzyme isolated by Yoshida *et al.* who found that neither of the amadoriases, isolated from *Aspergillus* and *Penicillium*, were active toward sarcosine (14).

Fructosyl propylamine was also tested as a substrate for sarcosine oxidase (obtained commercially from *Bacillus sp.*) in a reverse experiment. Sarcosine oxidase did not show any ability to turn over the substrate for Amadoriase I.

5.7 Does amadoriase return function to mildly glycated protein?

5.7.1 Preparation of a mildly glycated protein

From initial work (Chapter two), it was found that immediately following treatment of RNase A (20 mM amine) with methylglyoxal (10 mM), approximately 10% of lysine residues reacted (Figure 5.18). This degree of modification corresponds to around a 20% drop in activity of RNase A.

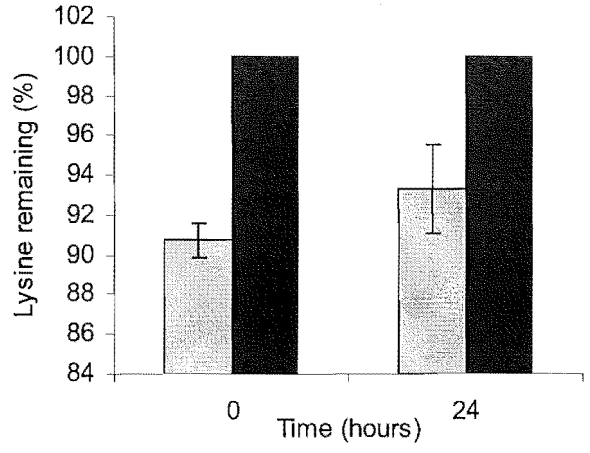


Figure 5.18: Percentage lysine remaining on incubation of RNase A with methylglyoxal. Grey bars depict methylglyoxal and RNase A incubations. Black bars represent RNase A only incubations. Percentages are calculated relative to the appropriate RNase A only time controls. Error is expressed as standard error of the duplicate experiments with duplication of each sample within the experiment.

These experiments were carried out in phosphate buffer due the fact that amadoriase required stabilisation during experiments. Substitution of water with phosphate buffer in incubations appeared to slow the break-down of RNase A in incubated controls over time (Chapter two).

5.7.2 Experimental approach to measuring amadoriase I activity

The stability of amadoriase I during early experiments

Initial attempts to determine whether amadoriase I returned activity to glycated RNase A encountered difficulties due to the unstable nature of amadoriase I. SDS-PAGE analysis of early experiments confirmed that amadoriase was breaking down during incubation at 25°C for 6 hours, prior to assay (Figure 5.19).

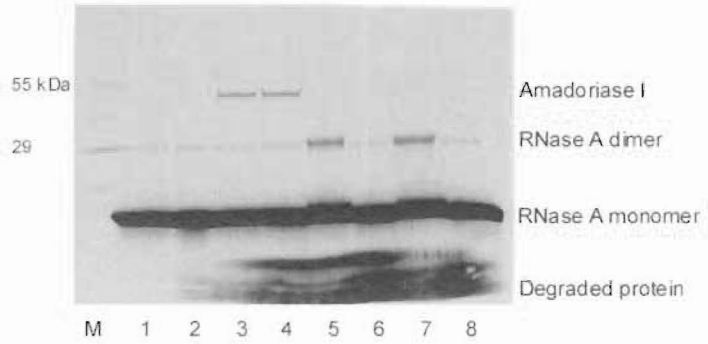


Figure 5.19: An 8-16% SDS-PAGE gel of incubations undertaken to assess amadoriase I stability during incubations. M, Marker; 1, methylglyoxal + RNase A time 0; 2, RNase A only time 0; 3, methylglyoxal + RNase A + amadoriase I time 0; 4, RNase A + amadoriase I time 0; 5, methylglyoxal + RNase A 1 h with subsequent incubation for 6 h at 25°C; 6, RNase A only 1 h with subsequent incubation for 6 h at 4°C; 7, methylglyoxal + RNase A + amadoriase I 1 h with subsequent incubation for 6 h at 25°C; 8, RNase A only + amadoriase I 1 h, with subsequent incubation for 6 h at 25°C.

This necessitated a change in conditions resulting in the incubation of the glycated substrate for longer time periods at a lower temperature (4°C). Figure 5.19 also shows an increase in dimer after the incubation periods with methyglyoxal and their intensities are equivalent in the absence (lane 5) or presence of amadoriase I (lane 7), relative to the appropriate controls (lanes 6 and 8). This demonstrates that only a

small degree of crosslinking of RNase A by methylglyoxal is occurring, as anticipated (Chapter two).

The modified method involved incubation of RNase A with methylglyoxal in phosphate buffer for 1 hour (to form the mildly glycated substrate). Amadoriase I was then added and the samples incubated at 4°C for 3 days. SDS-PAGE confirmed that amadoriase I was still present in incubation mixtures after 3 days at 4°C (Figure 5.20).

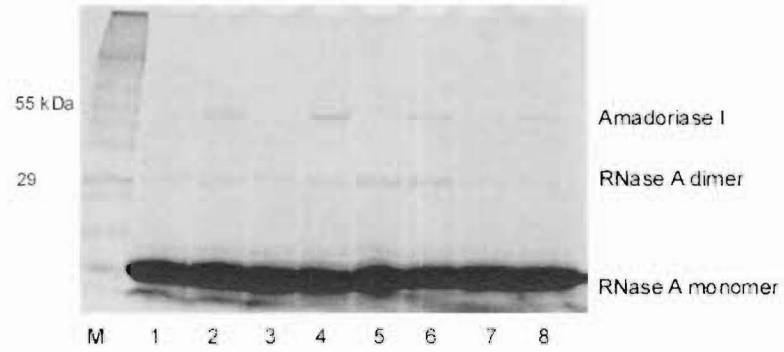


Figure 5.20: An 8-16% SDS-PAGE gel of incubations undertaken to assess amadoriase I stability during incubations. M, Marker; 1, methylglyoxal + RNase A time 0; 2, methylglyoxal + RNase A + amadoriase I time 0; 3, RNase A only time 0; 4, RNase A + amadoriase I time 0; 5, methylglyoxal + RNase A 1 h with subsequent incubation for 3 days at 4°C; 6, methylglyoxal + RNase A + amadoriase I 1 h with subsequent incubation for 3 days at 4°C; 7, incubated control 1 h with subsequent incubation for 3 days at 4°C; 8, incubated control + amadoriase I 1 h, with subsequent incubation for 3 days at 4°C.

The catalytic efficiency of RNase A following glycation and subsequent treatment with amadoriase I

Incubations were included where phosphate buffer was substituted for amadoriase to gauge the extent of glycation during the refrigeration period, and to provide a sample for direct comparison with those that contained amadoriase. To obtain a reference point to determine the extent of glycation over the initial 1 hour period of incubation, samples were prepared that had been frozen immediately on addition of methylglyoxal to RNase A (Amadoriase I or phosphate was also added at the time of freezing). The activity of RNase A was then measured. If the amadoriase I had been successful in deglycating RNase A, it was expected that RNase A, having been

inactivated on glycation by methylglyoxal, would have at least some of its activity restored.

Figure 5.21 shows that RNase following incubation for 1 hour at 37°C in the presence of 10 mM methylglyoxal, decreased in activity relative to the protein-only control incubated for the same time period (MR1 *vs.* IC1), confirming the results gained in Chapter four with higher concentrations of methylglyoxal. A significant difference in the mean activity of protein that had been incubated in the presence of methylglyoxal over time was noted, relative to those incubations that had not ($P<0.05$) (see Appendix three for ANOVA table). Critically, although addition of amadoriase I appeared to increase the activity of RNase A, the difference was not significant ($P=0.1$, Appendix three) (*e.g.* MR(A)1 *vs.* MR1).

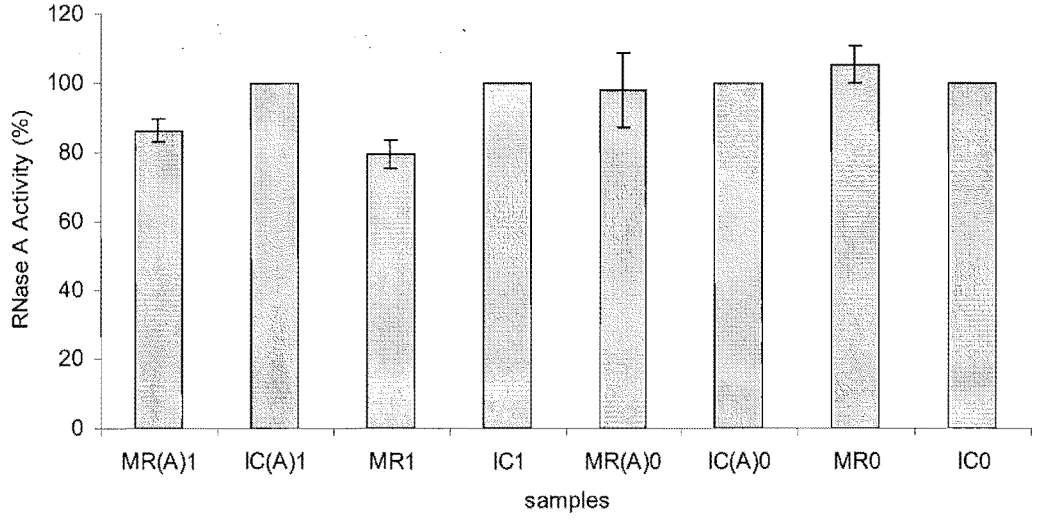


Figure 5.21: Percentage activity of RNase A in amadoriase I experiments. MR(A)1, methylglyoxal + RNase A incubated for 1 h with subsequent addition of amadoriase I; IC(A)1, RNase A only incubated for 1 h with subsequent addition of amadoriase I; MR 1, methylglyoxal + RNase A incubated for 1 h with subsequent addition of phosphate buffer; IC1, RNase A only incubated for 1 h with subsequent addition of phosphate buffer, MR(A)0, IC(A)0, MR0, IC0. Exactly as above for previous incubation but samples were frozen at time 0 instead of a 1 h incubation. Activity is expressed as a percentage relative to the appropriate controls and is an average of 4 experiments (with duplicate readings within each experiment). Error is expressed as standard error of the mean for 4 experiments.

The focal point of this chapter was to assess whether amadoriase I could effectively use mildly glycated protein as a substrate. The results reported here suggest that this was not possible, which may be due to a number of reasons. Firstly, the amadoriase I was incubated in the presence of possible unreacted methylglyoxal, which may have resulted in the glycation and subsequent inactivation of amadoriase I. From analysis of SDS-PAGE gels, it appears that the amadoriase I had not been crosslinked, but non-crosslinking methylglyoxal modifications may still exist. More critically, dialysis of the glycated sample to remove unreacted methylglyoxal and subsequent addition of amadoriase I did not appear to cause a difference in results, suggesting that this is unlikely.

Secondly, the amadoriase I may have not recognized the products from the reaction of methylglyoxal with RNase A-bound lysine, even in a mildly glycated state. Finally, if the production of a mildly glycated protein results in a small drop in activity, it can be difficult to determine whether any change on addition of amadoriase is indeed a true result or within experimental error of the experiment. Future work must be undertaken to investigate all of these possibilities.

Wu and Monnier have recently reported that charges on the surface of the protein amadoriase I preclude binding of amadoriase I to proteinaceous substrate (54). After amidation of the glycated protein carboxylate groups, amadoriase I is able to turn over glycated protein substrate (54). It is proposed that amidation changes the surface electrostatic interaction between substrate and enzyme.

5.8 Application of novel assay methods

The two novel methods outlined here were used in studies detailed in appendix four and five. The first study involved the time-resolved coupled assay, which was used to test the activity of two mutants of amadoriase I, H357N and S370A, (Appendix four). Both enzymes were found to be catalytically inactive by this method (Appendix four). Further work must be undertaken to establish whether the drop in activity is due to specific active site chemistry or protein misfolding.

The second assay method, involving the RNase A assay adapted from chapter four that was used in this chapter to assess amadoriase I activity on glycated protein, was employed experiments performed to isolate an enzyme that could act on glycated protein (Appendix five). Experiments outlined in appendix five examined isolates from raw milk for such enzymes, as milk is rich in many of the constituents of the Maillard reaction (Appendix five). From initial screening, it appeared that a Pseudomonad potentially possessed deglycating activity. Both assays developed in this chapter were performed to assess this deglycating activity, using a crude extract of this micro-organism. The time-resolved coupled assay used for assaying the catalytic activity of the amadoriase I mutants showed no activity in the crude using either fructosyl propylamine or the glycated casein as substrates. However, as glycated protein was the initial substrate used for culture of the Pseudomonad, the ability of the crude mixture to return activity to glycated RNase A was assessed. The second assay that was used to assess activity on glycated RNAse was employed to corroborate the results obtained using the time-resolved assay. Experiments using this assay showed that activity was not returned to glycated RNase A that was incubated with the crude mixture. These results suggest that the crude mixture did not contain an enzyme with deglycating ability.

5.9 General discussion and conclusions

This chapter has involved the development of two new assays to measure amadoriase activity in a variety of situations. The first, a time-resolved coupled assay to measure kinetic parameters of amadoriase I resulted in the generation of a K_m of 11 μM for fructosyl propylamine and a k_{cat}/K_m of $3.5x10^5$. These both differ with regard to the published values for this enzyme. The lower than previously published Michaelis constant may be due to the correct estimation of initial rate that is provided by the time-resolved assay developed here. Amadoriase I, with a K_m in the low micromolar range for fructosyl propylamine and a k_{cat}/K_m in the region of 10^5, has a high affinity for its substrate which is rapidly turned-over. This assay proved to be invaluable for purification and substrate specificity studies of amadoriase I. It has also aided the determination of the conditions in which amadoriase I is most stable for storage and experiments.

The second assay developed in this chapter, allowed for the determination of the direct effect amadoriase I had on the activity of a glycated protein. This approach provides more physiologically relevant information such as whether the function of the protein has been restored following incubation with amadoriase I. Previously published methods to ascertain the activity of amadoriase I on glycated protein have involved measurement of product formation or a decrease in substrate (4,6). These assays are unable to show what effect the amadoriase I has had on the substrate protein, beneficial or otherwise. Although amadoriase I did not restore function to RNase A, this method has provided a new tool to search for enzymes that can deglycate protein from other organisms. The inability of amadoriase I to act on protein treated with one of these dicarbonyls, methylglyoxal, is not advantageous from a therapeutic point of view if these are physiologically important species that modify protein.

In summary, this chapter has reported two novel assays to assess the activity of amadoriases under a variety of conditions. These have been successfully employed in site-directed mutagenesis studies and have provided useful tools to assess the deglycating ability of amadoriase I using glycated protein as a substrate and the search for amadoriase activity from a bacterium isolated from raw milk.

5.10 References

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Chapter Six

Summary and conclusions

This thesis sought to examine a wide range of aspects of the Maillard reaction, with an emphasis on the amino acids required for protein crosslinking, and the effect it had on the function of a model protein, ribonuclease A. Three α-dicarbonyls were selected to achieve this task: the physiologically relevant glyoxal and methylglyoxal, and the structurally related diacetyl. One of these, methylglyoxal, was used in later stages of this thesis, in studies undertaken to reverse the early stages of the Maillard reaction on proteins, using amadoriase I. To monitor this, two novel assays were successfully developed.

During the course of this thesis, diagnostic tools were developed to monitor the progression of the Maillard reaction. Chapter two assessed a number of methodologies for the experimental conditions required in this thesis. The initial approach, which measured the decrease in α-dicarbonyl concentration over time, met with limited success. This was attributed to the reactive nature of the α-dicarbonyl compounds. A measure of lysine concentration over time during incubation with the three α-dicarbonyls was more successful. A dramatic decrease in the lysine residues within RNase A following incubation with methylglyoxal was observed. A decrease in lysine residues was also observed for glyoxal and diacetyl, albeit at a much slower rate than methylglyoxal. The order of α-dicarbonyl reactivity (*i.e.* methyglyoxal >> glyoxal ≈ diacetyl) was mirrored by SDS-PAGE analysis, another method that was employed to measure the extent of the Maillard reaction. The extensive crosslinking observed on incubation of methylglyoxal with RNase A was almost immediate. Glyoxal and diacetyl again showed much less reactivity, with regard to crosslinking. The two methodologies for determining the extent of the Maillard reaction *in vitro*,

lysine analysis and SDS-PAGE, provided useful tools for the experiments that were undertaken in subsequent chapters.

The Maillard reaction has attracted a great deal of attention from those working in the medical arena. Central to this study is the effect that the products have on their target biomolecules such as circulatory, structural or enzymatic proteins. Little is known about the function of these proteins when they are crosslinked as advanced glycation end products. However, a class of inhibitors has been developed that is borne out of the assumption that cleaving protein crosslinks will return the protein to its native function. Furthermore, the amino acids required for the crosslinking process to occur have not been examined in detail. Chapters three and four sought to address the issue of specificity of the crosslinking reaction and the relationship of crosslinking to function of a model protein.

In this work, the amino acid requirements for crosslinking by the three α-dicarbonyls have been studied. Using carefully selected proteins, a lysine-free protein (that contained arginine) and an arginine-free protein (that contained lysine), the amino acid requirements for crosslinking were addressed. Interestingly, methylglyoxal only showed a capacity to crosslink the arginine-free peptide and could not crosslink the lysine-free peptide. Glyoxal however, could not crosslink the arginine-free peptide, but could crosslink the lysine-free peptide, which was later found to occur *via* the *N*-terminus of the protein. Diacetyl did not show any capacity to crosslink either protein, suggesting other routes to crosslink formation that account for the results observed during glycation of RNase A. These results demonstrate, for the first time, that either a lysine or an *N*-terminus is essential for crosslinking by α-dicarbonyls to occur. Also, arginine was not essential for crosslinking by α-dicarbonyls, under the experimental conditions employed in this work.

The underlying assumption that crosslinking will affect the function of a protein, has not been rigorously tested. From work detailed in this thesis it is evident that a loss in activity is observed during crosslinking of RNase A, with the three α-dicarbonyl compounds. However, it appears that inactivation of protein precedes crosslinking.

Thus, non-crosslinking modifications by α-dicarbonyls may be sufficient to inactivate protein, rendering crosslink breaking therapeutics ineffective.

As more becomes known about the complexities of the Maillard reaction, inhibitors have been designed at a variety of points in the reaction scheme. One class of inhibitors, the amadoriases, provides an enzymatic means for reversal of the early stages of the reaction. However, a difficulty has arisen that these cannot act on heavily glycated protein. Whether amadoriase I could act on mildly glycated was not known. Experiments undertaken in Chapter five sought to investigate this.

A bottleneck in characterisation of enzymes has been the development of an appropriate enzyme assay, and the amadoriases are no exception. Chapter five details the successful development of two novel assays to aid further characterisation of the amadoriases. The first assay was a time-resolved coupled assay and was used to determine the kinetic parameters of amadoriase I. Using this assay, a K_m of 11 μM, a (k_{cat}) of 3.5 sec^{-1} and a k_{cat}/K_m 3.25 x 10^5 M^{-1} sec^{-1} were obtained. All of these values differ to those that have been previously published and may reflect the determination of a true initial rate, rather than an underestimated initial rate, which can result from discontinuous assays that have been used in the past. This assay was successfully used to monitor the catalytic activity of two amadoriase I mutants, H357N and S370A. Both of these enzymes were found to be catalytically inactive following mutagenesis. Further studies need to be undertaken to determine whether these amino acids are critical for catalysis or that the mutations have caused the protein to misfold.

The second assay was devised to measure the activity of amadoriase I on glycated protein. Traditional assays have generally measured the formation of product or consumption of substrate. However, in the case of glycated protein, these do not show whether the substrate protein has had its activity restored after deglycation by amadoriases. Therefore, the assay outlined in Chapter five sought to address this by directly measuring the activity of glycated RNase A before and after treatment with methylglyoxal. It was hypothesised that after incubation with amadoriase I, at least some of the activity of RNase A would be restored. However, the results did not

show a return to function for RNase A following its treatment with amadoriase I that was greater than the error in the measurement.

Finally, the RNase A assay was used to search for an amadoriase-like enzyme from *Pseudomonas fluorescens* that had been cultured on glycated protein as a sole carbon source. However, the crude extract of this Pseudomonad did not show any such deglycating ability using either this assay, or the time-resolved assay that was used in the amadoriase I mutagenesis studies.

In summary, this thesis has successfully determined the amino acid requirements for protein crosslinking by α-dicarbonyls to occur and the effect crosslinking has on the function of a protein. The data presented here will inform the future design of Maillard inhibitors. Finally, the development of two novel assays to measure amadoriase activity has provided useful tools for researchers working in this burgeoning area.

Chapter Seven

Experimental

7.1 Materials

Unless otherwise stated, all compounds were purchased from either Sigma-Aldrich Pty. Ltd (Australia), Sigma-Aldrich Corp. (USA), BDH Laboratory Supplies, Invitrogen, Stratagene or Roche and were analytical grade. Solvents were generally purchased from BDH chemicals and were analytical grade.

RNase A (Type X-IIA, from bovine pancreas), renin substrate tetradecapeptide (porcine) and somatostatin (synthetic) were purchased from Sigma-Aldrich Corp. (U.S.A.)

Centrifugation of large volumes (>200 mL) was undertaken using a Heraeus Sepatech 20RS centrifuge using a Sorval rotor, GS-3 (6 x 500 mL fixed angle rotor, max *g*-force 13,680 x *g*). Centrifugation between 10-200 mL was undertaken using an Eppendorf 5403 centrifuge (6 x 85 mL fixed-angle rotor, max *g*-force 15,000 x *g*). The same centrifuge was employed to perform refrigerated centrifugation steps from 1 μL-200 mL of solution (30 x 1.5 mL fixed-angle rotor, max *g*-force 20,000 x *g*). RT spins under 1.5 mL were undertaken using a Heraeus Biofuge 13 or an Eppendorf Mini-Spin Plus centrifuge (12 x 1.5 mL fixed-angle rotor, max *g*-force, 14,000 x *g*).

8-16 and 4-20% SDS-PAGE gels were purchased from Gradipore (NSW, Australia). Electrophoresis was performed using either a Bio-Rad mini PROTEAN® 3 Cell gel kit or a Bio-Rad PROTEAN® II xi Cell kit in conjunction with either a Bio-Rad 300 or

1000 Powerpac unit. SDS-PAGE gel images were captured using a Nikon D1x digital camera fitted with a Nikon 60 mm macro lens.

Agarose gel electrophoresis was undertaken in a Hoefer HE33 Mini Horizontal Submarine Unit, in conjunction with a Bio-Rad 300 Powerpac unit. Agarose gels, following staining, were visualised with an Electrophoresis Documentation and Analysis System (Kodak Technologies), comprised of a Kodak digital camera and a Sigma T2210 UV transilluminator (302 nm), interfaced with a computer loaded with Kodak Digital Science 1D software.

Kinetic measurements were carried out using a Hewlett Packard 8452A or 8453 diode-array spectrophotometer interfaced with a personal computer loaded with UV-Visible Chemstation software (Agilent Technologies). Temperature controlled experiments were undertaken using a water bath attached to the spectrophotometer, the temperature was maintained by a Heidolph temperature controller.

Standard colorimetric assays were undertaken using either the above spectrophotometers or a Bio-Rad SmartSpec 3000.

All cuvettes, unless otherwise specified, were 1 cm path length plastic cuvettes from Sarstedt Inc., supplied by Global Science.

Unless otherwise stated, the pH of incubations was adjusted using Merck Universalindikator pH 0-14 strips. pH adjustment of buffers was performed using an UltraBasic Benchtop Meter UB-10 with the pH meter calibrated against pH standards of 4, 7 and 10. Some pH adjustment was also undertaken using the 718 STAT Titrino pH Stat (Metrohm, Switzerland) equipped with a magnetic stirrer. The pH stat was controlled by a computer running Tinet v.2.3 software.

For purification of amadoriase I, Pharmacia Hi-Trap Chelating HP columns were employed (Amersham Biosciences). The pumps used to load and elute the columns were Gilson Miniplus3 peristaltic pumps.

Lyophilisation was undertaken using a centrifugal freeze drier (Edwards High Vacuum Ltd.).

The pET 15b vector encoding the amadoriase I gene was a kind gift from Prof. Vincent Monnier (Case Western University, Cleveland, Ohio). The bacterial strain *E. coli* BL21(DE3)pLysS was kindly supplied by Dr J. Klena. (University of Canterbury). *E. coli* XL-1Blue supercompetent cells were obtained from Stratagene (Global Science, New Zealand). *P. fluorescens* for amadoriase-like enzyme study was isolated by Sarah Day (M.Sc. student, University of Canterbury).

Nuclear magnetic resonance spectrometry (NMR) was recorded on a Varian Unity 300 instrument operating at 300 MHz for ^{1}H nuclei at 23°C. Multiplicities are denoted as singlet (s), triplet (t), multiplet (m) and sextet (sext).

Melting points were recorded on an electrothermal melting point apparatus and are uncorrected.

Sonication was performed using a Sonics Vibracell sonicator fitted with a CV33 model probe.

Fluorescence measurements to detect fibril formation were made using a Varian Cary Eclipse fluorescence spectrophotometer interfaced with a PC loaded with Cary Eclipse version 2 operating software. Fibrils, on staining with Congo Red, were visualised using a cross-polarising microscope (J. Swift and Son, London) fitted with 45x objectives (NA 0.85).

Primers for amadoriase I mutagenesis were HPLC grade and purchased from Invitrogen (Australia). The QuickChange site-directed mutagenesis kit for the creation of amadoriase I mutants was purchased from Stratagene.

Sterilisation of glycated protein substrate was undertaken using an Ultra-lum UVC 515 Ultraviolet multilinker unit (254 nm) at 120,000 $\mu J/cm^2$.

7.2 Methods for experiments outlined in Chapter two

7.2.1 Preparation of model system incubations

Lysine and α-dicarbonyl incubations

The adjustment of pH, incubation, storage and controls for all incubations were exactly as described below for all the three α-dicarbonyls, methylglyoxal, glyoxal and diacetyl. All incubations were repeated at least in triplicate.

An equal vol (50 μL) of a 260 mM α-dicarbonyl solution (pH 7, adjusted with NaOH) was added to an equal vol (50 μL) of 260 mM lysine (pH 7, adjusted with NaOH) to give final equimolar concentrations of 130 mM α-dicarbonyl and lysine. The solution was incubated at 37°C for the prescribed period of time and stored at -20°C prior to analysis. Lysine only controls were prepared exactly as above, with substitution of dH_2O in place of α-dicarbonyl. One control was frozen immediately in liquid nitrogen, whilst the other was incubated with the α-dicarbonyl-containing samples and removed at the conclusion of the incubation. An α-dicarbonyl only control was prepared by substitution of dH_2O in place of lysine and incubated for the same time period as those containing lysine.

RNase A and α-dicarbonyl incubations

For each α-dicarbonyl, all incubations were prepared at least in triplicate. RNase A was solubilised in dH_2O to give a final concentration of 50 mg/mL (pH 7, adjusted with NaOH). To this, an equal vol of a 260 mM α-dicarbonyl solution (pH 7, adjusted with NaOH) was added to give final concentrations of 25 mg/mL RNase A (20 mM amine) and 130 mM α-dicarbonyl. The amine concentration of the protein was calculated by dividing the mass (25 mg) by the MW of RNase A (13680) and multiplying by 11 (10 free lysine groups and 1 *N*-terminus in RNase A). Solutions were incubated at 37°C for the prescribed period of time and stored at -20°C prior to analysis. RNase A controls were prepared by substitution of α-dicarbonyl with dH_2O. One control was frozen immediately in liquid nitrogen, whilst the other was incubated with the α-dicarbonyl-containing samples and removed at the conclusion of the

incubation. An α-dicarbonyl only control was prepared by the substitution of RNase A for dH_2O and incubated for the same time period as those containing RNase A.

For experiments where the effect of phosphate on the rate of crosslinking and lysine concentration was assessed, the above incubations and controls were prepared. However, final concentrations of 10 mM (instead of 130 mM above) methylglyoxal and 25 mg/ml RNase A were prepared. Also, 100 mM sodium phosphate, pH 7.4, was substituted where dH_2O was employed in previous incubations and controls.

7.2.2 Determination of α-dicarbonyl concentration in incubations prepared in 7.2.1

o-*Phenylenediamine assay (oPDA) (1)*

Construction of calibration curves

From a 500 μM standard solution of α-dicarbonyl, a range of concentrations were prepared in triplicate in dH_2O (Table 7.1, middle column). Each of these solutions was diluted 1:1 in 10 mM *o*-phenylenediamine (*o*PDA) prepared in 80% acetic acid (Table 7.1, right hand column), incubated at 50°C for 10 min and the absorbance of the sample recorded in a spectrophotometer blanked to dH_2O. A_{max} varied for each dicarbonyl. The methylglyoxal-containing test solution was measured at A_{320}, glyoxal at A_{318} and diacetyl at A_{328}. Controls where 80% acetic acid was substituted for α-dicarbonyl were undertaken to exclude the possibility that change in absorbance was due to colouration of *o*PDA itself. Absorbances were plotted against varying concentration and a calibration curve and R^2 value generated.

α-dicarbonyl	[α-dicarbonyl] prior to dilution in *o*PDA (μM)	[α-dicarbonyl] after dilution in *o*PDA (μM)
Methylglyoxal	50-400	25-200
Glyoxal	50-500	25-250
Diacetyl	50-300	25-150

Table 7.1: α-Dicarbonyl concentrations used for the *o*PDA assay

Determining α-dicarbonyl concentration of samples prepared in 7.2.1

Methylglyoxal, glyoxal and diacetyl-containing incubations were diluted to concentrations of 300 μM, 400 μM and 250 μM in dH_2O respectively. These solutions were prepared in triplicate, and further diluted 1:1 in 10 mM *o*PDA prepared in 80% acetic acid and assayed as outlined above. These concentrations were selected following inspection of the calibration curves prepared above. The above no-dicarbonyl controls were again included. Furthermore, controls that did not contain *o*PDA (80% acetic acid in place) were included to assess the interference of the α-dicarbonyl compounds and/or the products formed on the reaction of RNase A with α-dicarbonyl in the assay. α-Dicarbonyl concentration was then interpolated from the constructed calibration curve.

p-*Hydroxybenzoic acid hydrazide (HBH) assay (2)*

Two varying approaches to α-dicarbonyl estimation by the HBH methods are detailed in this section. These involved the use of two forms of the derivitising agent (Table 7.2).

Reagent	HBH reagent #1 [Final] (mM)	HBH reagent #2 [Final] (mM)
Trisodium citrate dihydrate ($Na_3C_6H_6O_7.2H_2O$)	20	50
$CaCl_2$	10	20
NaOH	300	500
Sodium sulfite (Na_2SO_3)	50	100
HBH	50	100

Table 7.2: Reagents and concentrations required for preparation of HBH.

Reagents were mixed in descending order in dH_2O due to solubility issues. The above HBH solutions were prepared fresh prior to assay and kept in the dark before each assay.

Due to the varying concentration of derivitising agent, each method involved differing ranges of α-dicarbonyl concentration. Each assay method will be outlined below.

Construction of calibration curves using HBH reagent #1

10 μL of a range of α-dicarbonyl concentrations (Table 7.3, middle column) were mixed with 5 mL of HBH reagent #1 to give the final concentrations quoted in the right hand column of Table 7.3.

α-dicarbonyl	[Initial α-dicarbonyl] (mM)	[Final α-dicarbonyl] (μM)
Methylglyoxal	5.5-33.4	11-66.8
Glyoxal	2.8-30.8	2.8-61.6
Diacetyl	4.6-32.4	9.2-64.8

Table 7.3: The range of α-dicarbonyl concentrations used for the HBH assay method #1

Samples were heated at 100°C for 5 min then cooled on ice. Once cool, samples were mixed and the A_{420} recorded in a spectrophotometer, blanked against dH_2O. dH_2O was substituted for α-dicarbonyl to exclude the possibility that change in absorbance was not due to colouration of HBH itself over time. Absorbances were plotted against varying concentration and a calibration curve and R^2 value generated.

Construction of calibration curves using HBH reagent #2

1 mL of a range of α-dicarbonyl concentrations (Table 7.4, middle column) were mixed with 2 mL of HBH reagent #2 to give final concentrations quoted in the right hand column of Table 7.4. The remainder of the assay and controls were carried out as outlined for the method employed for the HBH #1 reagent.

α-dicarbonyl	[Initial α-dicarbonyl] (pM)	[Final α-dicarbonyl] (pM)
Methylglyoxal	22.2-111	7.4-37
Glyoxal	14.2-128.5	4.7-42.8
Diacetyl	12-126	4-42

Table 7.4: The range of α-dicarbonyl concentrations used for the HBH assay method #2

Determining α-dicarbonyl concentration of incubation samples prepared in 7.2.1

The concentrations, following dilution in HBH, outlined in Table 7.5, were employed for each α-dicarbonyl for both variants of the HBH assay. Following inspection of calibration curves, appropriate concentrations of each α-dicarbonyl (after dilution in HBH) were selected for both HBH methods as outlined in Table 7.5

α-dicarbonyl	[Final] method #1 (μM)	[Final] method #2 (pM)
Methylglyoxal	55.6	37.1
Glyoxal	55.4	37.1
Diacetyl	55.6	37.1

Table 7.5: α-Dicarbonyl concentrations used for the HBH assay of samples prepared in 7.2.1

Incubation samples were prepared and measured as outlined for the calibration curve for each respective assay (method 1 or 2). A no sample control was again included as for the calibration curves. Controls that did not contain HBH were included to assess the interference of the α-dicarbonyl compounds and/or the products formed on reaction of RNase A with the α-dicarbonyl. A background reading, as mentioned for the calibration curves, was also taken. Experiments were repeated at least in triplicate.

7.2.3 Determination of protein concentration in RNase A-containing incubations prepared in 7.2.1 (3,4)

In some experiments, dialysis was required to remove any remaining unreacted α-dicarbonyl from incubations containing RNase A. This involved dialysing the sample against 1 L of cold (4°C) dH_2O O/N, with at least one change of fluid in 7,000 MWCO dialysis cups (Pierce, USA). Protein concentration was then quantified by a derivation of the method outlined in the Bio-Rad protein assay instruction manual, adapted by Reid (4).

Construction of a calibration curve

RNase A standards ranging from 20-120 μg/mL were prepared in dH_2O in triplicate by dilution of a 2 mg/mL RNase A stock solution (prepared in dH_2O). 80 μL of each standard was added to a cuvette containing 720 μL of a 41.7 μg/mL SDS solution and well mixed. The assay was initiated by addition of 200 μL Bradford reagent (Bio-Rad) and the cuvette thoroughly mixed and incubated for 6 min at RT. The A_{595} was recorded in a spectrophotometer blanked to the SDS solution-Bradford reagent mixture, with 80 μL of water in place of the incubation. Absorbances were plotted against varying concentration, and a calibration curve and R^2 value generated. Experiments were repeated at least in triplicate.

Determination of protein concentration in RNase A – containing incubations

RNase A was diluted in dH_2O in triplicate to an expected concentration (60 μg/mL in dH_2O) within the linear region of the calibration curve prepared above. 80 μL of sample was added to 720 μL of a 41.7 μg/mL SDS solution and the remainder of the assay undertaken as for the calibration curve. Concentration was interpolated from the calibration curve. Experiments were repeated at least in triplicate.

7.2.4 Quantification of lysine in samples prepared in 7.2.1 using o*-phthaldialdehyde (5,6)*

Construction of calibration curves

For construction of a lysine calibration curve, standards ranging from 0.125 to 1 mM (prior to addition to 1 mL of *o*PA) of lysine were prepared in dH_2O in triplicate (from a 1.2 mM lysine stock prepared in dH_2O). For RNase A, standards ranging from 0.5 to 3.5 mg/mL (prior to addition to 1 mL of *o*PA) in dH_2O were prepared in triplicate from a stock solution of 3.5 mg/mL RNase.

*o*PA reagent was prepared fresh immediately prior to assay as outlined in Table 7.6.

Reagent	Vol (mL)	[Final]
0.1 M Sodium metaborate ($NaBO_2.4H_2O$)	25	50 mM
20% SDS	2.5	1%
40 mg *o*PA	in 1 mL 100% MeOH	15 mM
100% Mercaptoethanol	0.1	0.2%
dH_2O	21.4	

Table 7.6: The preparation of *o*PA reagent. *N.B.*, solution was kept in the dark until required.

50 µL of sample was added to a cuvette containing 1 mL of *o*PA reagent, thoroughly mixed, and incubated for 2 min at RT. The A_{340} was recorded in a spectrophotometer blanked to dH_2O. Absorbances were plotted against concentration and a calibration curve and R^2 value generated.

Determination of amine concentration of RNase A incubated with α-dicarbonyl

Incubations containing RNase A, which had been dialysed O/N to remove any remaining unreacted α-dicarbonyl and the protein concentration determined, were diluted in dH_2O to a concentration of 2 mg/mL following inspection of the calibration curve. 50 µL of sample was added to 1 mL *o*PA, mixed, incubated and the absorbance recorded, as outlined for the calibration curve samples. Controls were included to assess the interference of the α-dicarbonyl compounds and/or the products formed on reaction of RNase A with the α-dicarbonyl. For these controls, *o*PA was omitted from the assay. Concentration was interpolated from the calibration curve. Experiments were repeated in triplicate.

Statistical analysis of incubations containing phosphate

The experiment undertaken to measure the effect of phosphate glycation of protein as measured by lysine concentration, was designed as a split plot experiment. The main factor for ANOVA was "Phosphate" which was at the plot level, with replicates at the subplot level ("Preparation") and nested (*i.e.* repeated measurements at this level – "Sub"). A second factor, "Time", was within this repeated measurement. There was a second level of nesting where a repeated measurement was taken (of each sample) to measure pipetting error within the assay procedure, thus introducing a second level

of nesting. A multi-way ANOVA was then performed in S-PLUS and the *P* values calculated manually where required.

7.3 Methods for experiments outlined in Chapter three

7.3.1 Preparation of low molecular weight protein + α-dicarbonyl incubations

Stock solutions were prepared by solubilising the protein in dH_2O to give a final concentration of 50 mg/mL (pH 7, adjusted with 0.1 M NaOH). In the case of *N*-acetylated renin substrate, solubilisation necessitated the addition of a minimal amount of DMSO. Aliquots of each solution were diluted 1:1 with a 260 mM α-dicarbonyl solution (pH 7, adjusted with 0.1 M NaOH) to give final concentrations of 25 mg/mL protein and 130 mM α-dicarbonyl. The resultant solution was incubated at 37°C for the prescribed period of time then stored at -20°C prior to analysis. Protein only controls were prepared as above with substitution of α-dicarbonyl with dH_2O with one frozen at time 0 in liquid nitrogen, and the other incubated with the α-dicarbonyl-containing incubations until the conclusion of the incubation. An α-dicarbonyl only control was prepared by substitution of protein with dH_2O and incubated for the same time period as those containing protein. Incubations were repeated in duplicate.

7.3.2 SDS-PAGE of low molecular weight proteins and RNase A incubated with α-dicarbonyl

The wells of a Gradipore pre-cast 4-20% SDS-PAGE gel (for low molecular weight protein work), 8-16% (for RNase A work), were first rinsed with dH_2O to remove any residual storage buffer. The gel was then set into the electrophoresis rig and the top tank filled with chilled (4°C) 1x tank buffer that had been diluted from a 5x stock (Table 7.7) until the electrodes were covered. The space surrounding the outside of the gel was filled with 1x tank buffer.

For low molecular weight proteins, the following method was undertaken. 7 μL of a 25 mg/mL protein sample was diluted in 7 μL of 2x treatment buffer prepared as outlined in Table 7.8. The sample gently mixed and then boiled for 2 min and the

entire vol of the sample loaded into the appropriate well. 7 μL of Sigma ultra-low molecular weight marker (Table 7.9) was added to 7 μL of 2x treatment buffer and boiled as described for samples.

Reagents	Amount
Trizma base	45 g
Glycine	216 g
SDS	15 g
Cold dH_2O (4°C)	3 L

Table 7.7: Preparation of 5 x Tank Buffer, pH 8.3 (as outlined in the Bio-Rad Protean® II xi Cell manual p. 39) (7). Stored at 4°C prior to use.

The procedure for treatment of RNase A samples was exactly as outlined for the low molecular weight proteins with the exception that a wide range marker was employed in place of the ultra low molecular weight marker (Table 7.9). Gels were electrophoresed at a constant voltage of 150 V for 1.5 h (for 8-16% gels) or 2 h (for 4-20% gels) at 4°C.

Reagents	Vol (μL)	[Final]
1 M Tris-HCl pH 6.8	125	27.8 mM
10% (w/v) SDS	2000	4.4%
100% Glycerol	1000	22.2%
100% 2-Mercaptoethanol	500	11.1%
1% (w/v) Bromophenol blue	125	0.3%
dH_2O	750	
Total	4.5	

Table 7.8: Concentrations required for preparation of 2x treatment buffer. Stored at -10°C.

Following electrophoresis, the gel was placed in an airtight container and covered with the Coomassie stain (0.1% Coomassie brilliant blue, 50% MeOH, 10% glacial acetic acid, filtered on preparation) (8) and placed on an orbital shaker for 2 h. After this time, the stain solution was removed and replaced with destain (5% methanol, 10% glacial acetic acid) (8), and shaken over a number of hours with at least one change of destain.

Proteins	M.W.(Da.)	Wide-Range [a]	Ultra-Low MW [b]
Myosin, rabbit muscle	205,000	X	
β-galactosidase, *E. coli*	116,000	X	
Phosphorylase b, rabbit muscle	97,000	X	
Fructose-6-phosphate kinase, rabbit muscle	84,000	X	
Albumin, bovine serum	66,000	X	
Glutamic dehydrogenase, bovine liver	55,000	X	
Ovalbumin, chicken liver	45,000	X	
Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36,000	X	
Carbonic anhydrase, bovine erythrocytes	29,000	X	
Triosephosphate isomerase, rabbit muscle	26,000		X
Trypsinogen, bovine pancreas	24,000	X	
Trypsin inhibitor, soybean	20,000	X	
Myoglobin, horse heart	17,000		X
α-Lactalbumin, bovine milk	14,200	X	X
Aprotinin, bovine lung	6,500	X	X
Insulin, chain B oxidised	3,496		X
Bradykinin	1,060		X

Table 7.9: The SDS-PAGE markers used for electrophoresis work. [a] Employed in work with RNase A. [b] Employed in work with renin substrate tetradecapeptide and somatostatin.

7.3.3 Congo red staining method for detection of fibrils in RNase A samples incubated with α-dicarbonyl (9)

Samples (~10 μL) of 25 mg/mL RNase A incubations containing α-dicarbonyl prepared as outlined in section 7.2.1, were fixed onto a poly-L-lysine-coated glass slide by drying at 37°C. This process was repeated to ensure a reasonable amount of protein was fixed onto the slide to visualise any fibrils generated from glycation (~40

μL final vol on slide). The slide was placed in working solution I prepared from solution A (Tables 7.10 and 7.11), for 20 min. The slide was then transferred to working solution II from stock solution B (Tables 7.10 and 7.11) for a further 20 min. The slide was then washed in two changes of a solution of 10% formaldehyde, 90% ethanol, which did not exceed 10 sec. Samples were then visualised under a cross-polarising microscope. Those that emitted a green birefringence under polarising light, were deemed to contain fibrils.

Solution A	Solution B
10 g NaCl	2 g Congo red
1 L 80% (v/v) Ethanol	10 g NaCl
	1 L 80% Ethanol

Table 7.10: Stock solutions required for Congo red fibril experiments. *N. B.* Solutions were prepared and stored at RT for 24 h prior to use. These were replaced every second month.

Solution I	Solution II
100 mL of Stock solution A	100 mL of Stock solution B
1 mL 1% (w/v) NaOH	1 mL 1% (w/v) NaOH

Table 7.11: Working solutions required for Congo red fibril experiments. *N. B.* Both working solutions were filtered following preparation, and as they had a limited shelf life, were used within 15 min.

7.3.4 Thioflavin T assay for detection of fibrils in RNase A samples incubated with α-dicarbonyl Adapted from Waterhouse (10)

RNase A incubations containing α-dicarbonyl were diluted to a concentration of 0.5 mM in dH_2O. 10 μL was added to a quartz cuvette containing 3 mL of Thioflavin T buffer (Table 7.12) (final protein concentration, 22.8 μg/mL, 1.66 μM). The cuvette was covered, inverted to thoroughly mix and incubated for 3 min. At the conclusion of incubation, the cuvette was placed in a fluorometer blanked to Thioflavin T buffer. The wavelength of excitation was 450 nm. The complete emission spectra were

recorded, over a range that included the wavelength of 482 nm. Both excitation and emission slits were set to 5 nm.

Reagent	Amount	[Final]
1 mM Thioflavin T (31.89 mg/100 mL dH_2O, stored in the dark at 4°C)	500 µL	5 µM
Trizma base	0.61 g	50 mM
NaCl	0.59 g	100 mM
dH_2O	99.5 mL	

Table 7.12: Preparation of Thioflavin T buffer (pH 7.5, adjusted with conc. HCl). *N. B.* The solution was prepared fresh each experiment and where possible kept at 4°C in the dark.

7.4 Methods for experiments outlined in Chapter four

7.4.1 RNase A activity assays (11)

The RNase A assay employed here required the preparation of two buffers: MOPS buffer (Table 7.13) and methylene blue buffer.

Reagent	Amount	[Final] (mM)
MOPS	29.03 g	100
$Na_2EDTA{\cdot}2H_2O$ (0.5 M)	4 mL	2

Table 7.13: Preparation of MOPS buffer. *N. B.* The EDTA was added to the prepared MOPS (that had been adjusted to a pH of 7.4 using 10 M NaOH), and the pH re-checked and adjusted where required.

Methylene blue buffer was prepared by dissolving 1 mg of methylene blue in 100 mL of MOPS buffer. The methylene blue buffer was prepared fresh prior to each assay and adjusted to 0.5 ± 0.01 A.U. at 688 nm in a spectrophotometer blanked to dH_2O. As the solution was light sensitive, the buffer was kept in the dark during the assay.

Construction of a calibration curve to determine RNA concentration and assays to determine that enzyme concentration is proportional to rate

A series of RNA standards were constructed ranging from 0.25-10 mg/mL in MOPS buffer. The volumes outlined in Table 7.14 were dispensed into a cuvette:

Reagent	Vol(μL)	[Final] (μg/mL)
RNA standard (0.25-10 mg/mL)	100	25-1000
Methylene blue buffer	900	

Table 7.14: Volumes required to construct an RNA calibration curve

Each cuvette was incubated for 10 min in the dark at 25°C. The A_{688} was recorded in a spectrophotometer blanked to dH_2O. Each concentration was prepared and recorded in triplicate and the experiment repeated in duplicate. No-substrate controls were included which involved the substitution of RNA with MOPS buffer.

To confirm that rate was proportional to enzyme concentration the volumes outlined in Table 7.15 were dispensed into a cuvette, with the exception of RNase A.

Reagent	Vol (μL)	[Final] (μg/mL)
RNA (10 mg/mL)	60	600
Methylene blue buffer	940	
RNase A standard (5-60 μg/mL, prepared in MOPS buffer)	10	0.05-0.6

Table 7.15: Volumes required to determine if enzyme concentration was proportional to rate

Each cuvette was incubated at 25°C for 10 min in the dark. Then, 10 μL of an RNase A standard was added, mixed and incubated for 1 min. After this time, the ΔA_{688} was recorded for 120 sec. Linearity of time traces over this period was ensured and the concentration of RNA was calculated from the calibration curve prepared above. All RNase A standards were prepared in triplicate and the experiment repeated at least in triplicate.

Controls without enzyme and substrate respectively were included as controls to ensure that RNase A was solely responsible for the change in rate, and that the enzyme was not causing a rate by reaction with any other compound present in the cuvette.

Attempts at determining K_m *and* V_{max} *of RNase A* via *the methylene blue assay*

For this experiment, RNase A concentration was held constant and the amount of RNA varied as outlined in the Table 7.16.

Reagent	Vol (μL)	[Final] (μg/mL) (2 s.f.)
RNA standard (0.25-12 mg/mL)	100	25-1200
Methylene blue buffer	900	
RNase A (15 μg/mL, prepared in MOPS buffer)	10	0.15

Table 7.16: Volumes of reagents required to determine K_m and V_{max} of RNase A.

Incubation and measurement times and rate calculations were undertaken exactly as outlined for the experiments to determine if enzyme concentration was proportional to rate. All RNA concentrations were prepared in triplicate and the experiment repeated at least in triplicate. No-enzyme and no-substrate controls were again included.

Preparation of RNase A + α-dicarbonyls in the presence of Maillard inhibitors.

Incubations of RNase A, α-dicarbonyl and inhibitor (either aminoguanidine or 3,5-dimethylpyrazole-1-carboxamidine), were prepared as outlined in section 7.2.1., with the inclusion of Maillard inhibitors. Aqueous solutions of the α-dicarbonyls (390 mM) and inhibitors (390 mM) were prepared, and a 75 mg/ml solution of RNase A. Equal volumes of each were pipetted into to Eppendorfs to give final concentrations of 130 mM α-dicarbonyl and inhibitor, and 25 mg/mL protein. Controls were prepared as outlined in section 7.2.1. Where DMSO was required for solubilisation of aminoguanidine, this was included in the appropriate incubation controls.

The influence of α-dicarbonyls and Maillard inhibitors on the catalytic efficiency of RNase A.

RNase A incubations containing α-dicarbonyl and/or inhibitor was diluted to a concentration of 15 μg/mL. The following volumes were dispensed into a cuvette with the exception of the sample to be tested.

Reagent	Vol (μL)	[Final] (μg/mL)
RNA (10 mg/mL)	60	600
Methylene blue buffer	940	
Sample - RNase A (15 μg/mL, in MOPS buffer)		
± α-dicarbonyl	10	0.15
± inhibitor		

Table 7.17: Volumes of reagents required to test the influence of α-dicarbonyls and Maillard inhibitors on the catalytic efficiency of RNase A.

Incubation, sample addition, measurement times and rate calculations were the same as outlined for K_m and V_{max} experiments with the sample RNase A added to initiate the assay. Incubations were measured in triplicate with at least duplication of the experiment. No-enzyme and no-substrate controls were included in all experiments. In some cases, the solubilisation of Maillard inhibitors, such as aminoguanidine, necessitated the addition of solvents such as DMSO. The controls were adjusted accordingly (*e.g.* addition of a volume of DMSO to the reaction mix, equivalent to that in the RNase A sample) to ensure solvent was not interfering with the assay.

7.5 Methods for experiments outlined in Chapter five

All strain genotypes, media preparation and antibiotic concentrations are outlined in Appendix six.

7.5.1 Preparation of glycerol freezes of bacterial strains (12)

A colony selected from an LB-Agar plate (Appendix six), supplemented with the appropriate antibiotics, was used to inoculate a 3 mL solution of LB (Appendix six) containing the appropriate antibiotics. This was incubated O/N in a 37°C shaking water bath. Following incubation, 750 μL of culture was aliquoted into a sterile Eppendorf and centrifuged at 7,000 rpm for 2 min. The supernatant was discarded and the pellet resuspended in a further 750 μL of O/N culture and an equal volume of sterile 30% glycerol, mixed well, and stored at -80°C. This was carried out in triplicate to ensure the appropriate strain had been selected.

7.5.2 Preparation of competent cells for transformation with plasmid DNA encoding amadoriase I (12,13)

An O/N culture of *E. coli* BL21(DE3)pLysS (400 μL), supplemented with 30 μg/mL Cam, was used to inoculate a 20 mL solution of LB containing 30 μg/mL Cam and grown to an O.D. of 0.4 at 600 nm (measured in a spectrophotometer blanked to LB). When the O.D. was reached, the culture was immediately placed on ice. On cooling, the culture was centrifuged at 7,000 rpm for 3 min at 4°C (these centrifugation conditions were used for all subsequent steps). The supernatant was discarded and the pellet resuspended in 20 mL of cold sdH_2O (4°C), centrifuged and the supernatant discarded. The pellet was resuspended in 10 mL of cold sdH_2O (4°C) and centrifuged and the supernatant discarded. The pellet was gently resuspended in the residual supernatant and poured into sterile Eppendorfs and centrifuged. The supernatant was then gently removed and discarded and the pellet resuspended in 160 μL of cold sdH_2O (4°C). Cells were stored on ice for immediate transformation experiments or stored at -80°C.

7.5.3 Transformation of E. coli *BL21(DE3)pLysS or* E. coli *XL1-Blue with the vector containing amadoriase I (12,13)*

Competent cells (40 μL), prepared using the above protocol or purchased (in the case of *E. coli* XL1-Blue), were added to a UV-sterilised electroporation cuvette. 1 μL of

plasmid DNA (1-100 ng/mL, pET 15b vector containing amadoriase I gene) was added and electroporated at 1.8 kV in a 0.1 cm gap cell using an Electroporation GenePulser™ (Bio-Rad). The slide chamber and cuvettes were stored on ice prior to use. In parallel, sdH_2O was substituted for DNA and electroporated as described above. Following electroporation, 800 µL of SOC media (Appendix six) was added to the cuvette, gently mixed and transferred into a sterile Eppendorf and incubated for 1.25 h at 37 °C. After incubation, 100 µL of sample was spread onto LB-Agar plates supplemented with either Cam (30 µg/mL) and Amp (100 µg/mL) for *E. coli* BL21(DE3)pLysS or Tet (20 µg/mL) and Amp (100 µg/mL) for XL1-Blue and incubated O/N at 37°C. The remainder of the samples were centrifuged at 7,000 rpm for 2 min. at RT. As much of the supernatant was decanted and discarded and the pellet was resuspended in the residual solution. These suspensions were plated onto LB-Agar plates with the appropriatly supplemented antibiotics as outlined above. Six transformant colonies were selected from each plate and re-streaked individually onto fresh LB agar plates supplemented with appropriate antibiotics according to the strain in use and again incubated O/N at 37°C. O/N cultures were prepared from colonies selected from the plates and glycerol freezes prepared and stored as outlined in section 7.5.1. These transformed strains were referred to as *E. coli* BL21(DE3)pLysS(pET15b) or *E. coli* XL1-Blue(pET15b).

7.5.4 Plasmid mini-preparation of pET 15b vector via *alkaline lysis method (12)*

O/N culture (1.5 mL) was added to a sterile Eppendorf and centrifuged at 7,000 rpm for 90 sec, the supernatant discarded and the pellet resuspended in 100 µL of solution I (Table 7.18). On solubilisation of the pellet, 200 µL of solution II (Table 7.18) was added and the Eppendorf gently inverted and rolled until the mixture became viscous. Finally, 150 µL of solution III (Table 7.18) was added and the tube gently mixing and rotated and the flocculent broken up. The resulting mixture was stored on ice for 10 min then centrifuged at 11,000 rpm for 10 min at RT. The supernatant was poured into a sterile Eppendorf containing 2 vol of cold (-20°C) 100% EtOH, mixed and cooled on ice for 10 min and the pellet discarded. Following incubation on ice, the solution was centrifuged at 11,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was aspirated and the pellet washed with 500 µL of 70% RT EtOH and

re-spun at 11,000 rpm for 2 min. The supernatant was discarded and the pellet air-dried. The dried pellet was then resuspended in 40 µL of sdH_2O or TE buffer (10 mM Tris.HCl, 1 mM $Na_2EDTA{\cdot}2H_2O$ (pH 8, autoclaved) and stored at -10°C until further required.

Solution I	Solution II	Solution III
50 mM Glucose	0.2 M NaOH	60 mL 5 M KOAc
25 mM Tris-HCl (pH 8)	1% SDS	11.5 mL Glacial acetic acid
10 mM $Na_2EDTA{\cdot}2H_2O$ (pH 8)		28.5 mL dH_2O

Table 7.18: Solutions required from plasmid DNA preparation. Solutions I and III were prepared and autoclaved and stored at 4°C until required. Solution II was prepared immediately before each plasmid preparation and stored at RT.

7.5.5 Preparation of sequence quality plasmid DNA Adapted from Sambrook (12,13)

A 50 mL O/N culture of *E. coli* XL1-Blue(pET15b) supplemented with the appropriate antibiotics was centrifuged at 7,000 rpm for 2 min at RT and the supernatant discarded. The pellet was resuspended in solution I, and on solubilisation of the pellet, 5 mL of solution II was added and the tube gently inverted and rolled until the mixture became viscous. Finally, 5 mL of solution III was added and the tube gently mixed and rotated and flocculent broken up. The resulting mixture was stored on ice for 10 min, then centrifuged at 11,000 rpm for 10 min at RT. The supernatant was transferred to a sterile centrifuge tube containing 0.54 vol of isopropanol and stored in ice for 10 min and the pellet discarded. The solution was centrifuged at 11,000 rpm for 10 min at 4°C. The DNA pellet was rinsed in 1 vol of RT 70% EtOH and centrifuged and dried as previously described for the DNA mini-preparation (section 7.5.4).

To remove high molecular weight RNA, the pellet was redissolved in 1 mL of cold TE buffer and iced for 30 min. 1 mL of cold (4°C) 5 M lithium chloride (21.2 g LiCl in dH_2O 100 mL, autoclaved) was then added and the resulting solution iced for between 10-30 min. The solution was then centrifuged at 11,000 rpm for 10 min at

RT and the supernatant transferred into a fresh centrifuge tube containing 0.54 vol isopropanol. The solution was iced for 10 min and spun at 11,000 rpm for 10 min at 4°C. The pellet was again washed, centrifuged and dried as outlined in section 7.5.4.

To further remove RNA and any residual protein, the DNA pellet was resuspended in 200 µL of TE buffer and transferred to an Eppendorf containing 2 µL of RNase (10 mg/mL, DNase free) and incubated at 37°C for 30 min. 200 µL of phenol and 200 µL chloroform:isopropanol (24:1) was then added to the solution and gently mixed and centrifuged at 13,000 rpm for 5 min at RT. After centrifugation, the top layer (~180 µL) was transferred into a new Eppendorf containing 180 µL of chloroform:isopropanol (24:1). The solution was mixed and re-spun at 13,000 rpm for 5 min at RT. The top layer (~160 µL) was again transferred to a new Eppendorf containing 20 µL ($1/8^{th}$ vol) of 2 M potassium acetate in 2 vol of 100% (-20°C) cold EtOH. This solution was gently mixed and cooled on ice for 10 min and then spun at 13,000 rpm for 10 min at 4°C. DNA was rinsed, centrifuged and air-dried as described for the mini-preparation. The dried DNA was then resuspended in 20 µL of sdH_2O or TE.

7.5.6. Restriction analysis of plasmid DNA (12)

Typically, the following mixture was prepared following assessment of DNA concentration by either gel electrophoresis or 260:280 extinction coefficient readings (12).

Digest	x µL DNA (0.5 to 5 µL DNA in sdH_2O)
	1.5 µL 10x buffer (compatible with both restriction enzymes)
	0.2 µL *Bam*HI (10 units/µL)
	0.2 µL *Nde*I (10 units/µL)
	x µL sdH_2O (to adjust to the desired final volume)
Final volume	15 µL

This mixture was incubated at 37°C for 1-1.5 h.

Following digestion, samples were treated with 2.5 µL loading buffer (Table 7.19, left hand column) and loaded onto a 1% agarose gel (0.3 g in 30 mL in 1x TAE, diluted

from 50x TAE, right hand column of Table 7.19), along with 5 μL of marker (λ cut with *Hin*dIII). The gel was electrophoresed at 80 V for ~1.5 h in 1x TAE at RT. The gel was stained for 10 min in ethidium bromide and visualised on a UV transilluminator and photographed with a digital camera interfaced with a computer loaded with imaging software.

Reagents	[Final]	Reagents	Amount
100% Glycerol	30%	Trizma base	242 g
1% Bromophenol blue	0.25%	0.5 M EDTA pH 8.0	100 mL
1% Xylene cyanol	0.25%	Glacial acetic acid	57.1 mL
RNase (DNase free)	10 μg/mL	dH_2O	1 L

Table 7.19: Solutions required for DNA agarose electrophoresis. Loading buffer prepared and stored at -20°C (left hand column), and 50x TAE, pH 8 stored at 4°C, (right hand column).

The marker for agarose electrophoresis, λ cut with *Hin*dIII, has fragment sizes of 23.1, 9.4, 6.6, 4.4, 2.3, 2.1 and 0.5 (kb).

7.5.7 PCR site-directed mutagenesis of amadoriase I

Two mutants, H357N and S370A were prepared in collaboration with Andrea Uhlmann and Stephan Hegge respectively, according to the protocol outlined in the Quikchange site-directed mutagenesis kit instruction manual (14-16).

Primer design for the generation of H357N and S370A mutants

The primers for PCR were designed to meet the guidelines stipulated by Stratagene for successful mutagenesis (14). These were that:

- Both primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid.
- The primers were between 27 and 37 bases in length
- The primers had a calculated melting temperature (T_m) of greater than 78°C. ($T_m = 81.5 + 0.41(\%GC) - 675/N - \%$ mismatch, N is the primer length in base pairs).

- The base changes were in the middle of the primer with at least 10–12 bases of correct flanking sequence
- Finally, the primers had a minimum GC content of 40% and terminated in one or more CG bases.

The mutant primer T_m were 78.2°C and 81.51°C for the H357A and S370A mutants respectively and met all the other 4 requirements noted above.

PCR reaction conditions

For each mutant, a PCR reaction in thin-walled PCR tubes, was set up as described in Table 7.20. This has been shown for only the S370A (the conditions for H357N were exactly the same). A diagnostic control for this step was the reversion mutation of pWhitescript with mutagenic primers, which were supplied with the mutagenesis kit (Table 7.21). The pWhitescript plasmid has a point mutation in the *lacZ'* gene (β-galactosidase) generating a stop codon at the ninth position of the coding region. Mutagenic primers replace the stop codon with a glutamine codon restoring activity to the enzyme and allowing the determination of mutational efficiency by the proportion of blue/white colonies on IPTG/X-gal plates (Appendix six).

Reagents	Vol (μL)	Final Amount (ng)
10× reaction buffer	5	
Template dsDNA 7.2 kb plasmid	10	50
S370A primer #1	15	125
S370A primer #2	15	125
dNTP mix	1	
Double-distilled water (ddH_2O)	4	
*Pfu*Turbo DNA polymerase (2.5 Units μL^{-1})	1	

Table 7.20: Volumes required for the amplification of the S370A mutant.

Reagent	Vol (μL)	Final Amount (ng)
10× reaction buffer	5	
pWhitescript 4.5-kb control plasmid	2	10
Oligonucleotide control primer#1 34-mer	1.25	125
Oligonucleotide control primer#2 34-mer	1.25	125
dNTP mix	1	
Double-distilled water (ddH_2O)	37.5	
*Pfu*Turbo DNA polymerase (2.5 Units μL^{-1})	1	

Table 7.21: Vols. required for the control PCR reaction

Each reaction was overlaid with mineral oil to prevent evaporation and the PCR reaction carried out in a thermocycler equipped with a "hot bonnet", which also helped to prevent evaporation. Reactions were then cycled through PCR programmes outlined in Table 7.22.

Control reaction			Sample reaction		
Step	Temperature (°C)	Time (min)	Step	Temperature (°C)	Time (min)
1	95	0.5	1	95	0.5
2	95	0.5	2	95	0.5
3	55	1		55	1
4	68	12		68	7.2
5	steps 2–4 eleven times			steps 2–4 twelve times	

Table 7.22: PCR cycling parameters for control and mutant reactions

At this point, agarose gel electrophoresis was used to check for amplification of the parent plasmid using the equivalent amount of starting template as a control.

After amplification, endonuclease enzyme, *Dpn*I (1 μL, 10 Units μL^{-1}), was added to each of the reactions below the mineral oil. *Dpn*I is a methylated and hemimethylated specific enzyme, which digests the parent template DNA, leaving the mutation-containing newly synthesised DNA intact. The reaction mixture was gently, but

thoroughly mixed, centrifuged, and incubated at 37°C for 1 h to digest the parental (non-mutated) supercoiled dsDNA.

Transformation of E. coli *XL1-Blue with mutated plasmids and DNA sequencing*

Following mutagenesis, each of the plasmids were used to transform *E. coli* XL1-Blue supercompetent cells (Stratagene). Competent cells were gently thawed on ice and aliquots (50 µL) transferred to pre-chilled Falcon 2059 polypropylene tubes. DNA (1 µL) was added to separate aliquots, mixed gently by pipetting and incubated on ice (30 min). The transformation reaction was heat pulsed for 45 seconds at 42°C, and then placed on ice for 2 min. NZY+ broth (Appendix six) (0.5 mL, preheated to 42°C) was added and incubated (37°C for 1 h) with shaking at 225–250 rpm. Plasmid DNA was then prepared from each respective strain, *E. coli* XL1-Blue (pET 15bS370A) and *E. coli* XL1-Blue (pET 15bH357N) as outlined in section 7.5.5.

DNA sequencing of the above mutants was performed at the Waikato Sequencing Facility. Plasmids to be sequenced were prepared to the highest quality, quantified spectrophotomectrically (12), and sequenced using the dideoxynucleic acid chain termination method (17), using T7 promoter and T7 terminator primers, along with the custom primer outlined below. The custom primer was to ensure a complete sequence where readthrough from where the T7 promoter would not cover this area of sequence. The primer below met the following sequencing requirements as stipulated by the DNA sequencing facility (denoted in parentheses are the properties of the below primer): between 18-30 bases (24 bases), GC content 40-60% (50%), T_m = 55-75 °C (66.77°C), A-T rich at the 3' end.

5' **1002** GC GCA CGA GAC TTT CTC CAT GAT A **1025** 3'

Bold denotes the base pair number within the amadoriase I gene.

Restriction analysis of S370A pET15b plasmid

Restriction analysis was undertaken as outlined for the wild-type plasmid outlined in section 7.5.6, with substitution of *Nde*I for *Nae*I. Electrophoresis was undertaken as outlined for the wild type with a 1.5% agarose gel used in place of a 1% gel due to the formation of low molecular weight fragments (160-400 bp). A 1kb marker was used,

along with λ cut with *Hind*III, which contained fragment sizes: 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2 and 0.1 kb.

Transformation of E. coli *BL21(DE3)pLysS with mutated plasmids*

Following sequencing, the plasmid DNA of each mutant was used to transform *E. coli* BL21(DE3)pLysS to create two strains, *E. coli* BL21(DE3)pLysS(pET 15bS370A) and *E. coli* BL21(DE3)pLysS(pET 15bH357N), for expression purposes. The preparation of competent cells and transformation protocols were undertaken as outlined in sections 7.5.2 and 7.5.3.

7.5.8 Over-expression of amadoriase I isozyme from E. coli *(BL21)DE3pLysSpET15b Adapted from Wu* et al. *(18,19)*

Harvest of E. coli *BL21(DE3)pLysS pET15b and purification of amadoriase I*

25 mL of an O/N culture *E. coli* BL21(DE3)pLysSpET15b was used to inoculate 1 L of LB supplemented with the Cam (30 μg/mL) and Amp (100 μg/mL). The inoculated culture was then incubated at 37°C on a shaking platform until the O.D. reached between 0.4-1.0 (19). Amadoriase I expression was then induced by adding 1 mL of 0.25 M IPTG (in dH_2O, filter sterilised) to the culture and incubated for a further 3-4 h. After incubation, the culture was immediately cooled in ice. Cells were then harvested by centrifugation at 5,000 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 1 vol of ice cold (4°C) lysis buffer (Table 7.23). Cells were again centrifuged at 5,000 x *g* for 10 min at 4°C, the supernatant discarded and the pellet resuspended in half a vol of lysis buffer and the cells centrifuged as described immediately above. The pellet was resuspended in a minimal volume of ice cold lysis buffer and all cells were pooled into a 50 mL centrifuge tube, which was then centrifuged as above. The supernatant was discarded and the pellet was flash frozen in liquid N_2 and thawed O/N at 4°C on ice. The pellet was resuspended in around 30 mL of ice cold (4°C) lysis buffer and sonicated on ice in 30 sec bursts until the A_{260} of the extract no longer increased. The extract was centrifuged at 13,000 x *g* for 10 min at 4°C, the pellet discarded and the supernatant passed through a 0.45 μM PVDF filter.

Lysis Buffer pH 8	Wash Buffer pH 8	Elute Buffer pH 8
20 mM NaH_2PO_4	20 mM NaH_2PO_4	20 mM NaH_2PO_4
500 mM NaCl	500 mM NaCl	500 mM NaCl
20 mM Imidazole	40 mM Imidazole	100 mM Imidazole

Table 7.23: Buffers used during the purification of amadoriase I. All were prepared fresh before each purification and stored at 4°C. All buffers were filtered through a 0.45 μM PVDF filtered prior to use with the His-Trap columns.

The supernatant was loaded onto two Pharmacia His-Trap columns that had been connected together, pre-charged with Ni^{2+} and equilibrated with lysis buffer at a flow rate of 0.5 mL/min (as described by the manufacturer). The columns then washed with 30 mL of wash buffer (Table 7.23). Following the washing step, the protein was eluted from the column in 1 mL fractions using elute buffer (Table 7.23). These fractions, along with the load and wash, were subjected to SDS-PAGE and activity measurements. Active, pure fractions (those with a rate above $1x10^{-3}$ for 20 μL of sample as judged by a coupled assay (section 7.5.10) at 5-10 x K_m of fructosyl propylamine and a single band visible by SDS-PAGE, see below) were pooled and dialysed against 3 L of 10 mM sodium phosphate buffer (pH 8) for 2 h at 4°C using Sigma 12,000-14,000 MW cut-off tubing (Diameter = 16 mm, capacity, 60 mL/ft, prepared as according to the manufacturer's instructions) with at least one change of 1.5 L of 10 mM sodium phosphate buffer (pH 8). The resulting retentate was tested for activity and protein concentration determined (see below), then stored at -80°C. amadoriase I was found to lose approximately half its activity after storing at -20°C for 24 h amadoriase I stored at -80°C displayed activity similar to that stored at 4°C after 24 h.

SDS-PAGE analysis of amadoriase I purity during purification experiments

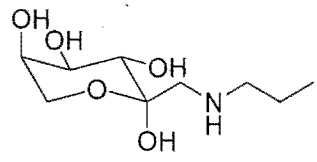
SDS-PAGE experiments to determine the purity of amadoriase I fractions were undertaken using exactly the same system outlined for RNase A in section 7.3.2. The only differences being the amount of sample analysed, with 20 μL of load and wash (incubated with 20 μL 2x treatment buffer) loaded onto the gel. 5 μL of crude was added to 5 μL 2x treatment buffer.

Estimation of protein concentration during purification of amadoriase I

The assay method is essentially that outlined for RNase A in section 7.2.3 with BSA used as a reference protein in place of RNase A. A 2 mg/mL solution of BSA was diluted to the same standard concentrations as RNase A (*i.e.* 20-120 μg/mL) in dH_2O. The 41.7 μg/mL solution of SDS used in RNase A experiments was substituted with dH_2O in this work. Triplicate series of dilutions of the sample under study were diluted to a concentration in the linear range of the BSA calibration curve and assayed as outlined in section 7.2.3.

Samples containing protein were diluted in dH_2O until they gave an absorbance that lay in the region of linearity, as defined by the above calibration curve. The concentration was interpolated from the curve.

7.5.9 Synthesis of fructosyl propylamine (1-amino-1-deoxy-D-fructose) (20)



1-amino-1-deoxy-D-fructose

Fructosyl propylamine was prepared as follows: 15 g (0.083 mol) of glucose and 20 mL of propylamine (0.237 mol) were heated to 70°C and stirred until as much of sugar as possible was dissolved. After 10 min, the product dissolved and became white-yellow and hardened to a gel-like consistency. The mixture was diluted in 200 mL of isopropanol and poured into 800 mL of diethyl ether. The precipitate was filtered and dissolved in 300 mL of boiling dioxane. To this, 20 g of oxalic acid dissolved in 200 mL of dioxane was added, and the mixture was heated for 15 min at 70°C. The resulting precipitate was crystallised from dioxane/methanol (1:1). The oxalate was replaced by chloride using anion exchange chromatography (Sephadex) eluted with dH_2O. Fractions were dried by either rotary evaporation or by lyophilisation. A crystalline light brown product was yielded from this protocol (470 mg, 3.13%).

Melting points

Salt	Literature	This work
Oxalate	138°C (21) 118-120°C (22)	133-135°C
Chloride	?	135-140°C

Table 7.24: Literature and observed melting points of fructosyl propylamine

^{1}H NMR data has been reported previously for the chloride salt (23), and these results are in agreement with those obtained by this procedure.

^{1}H NMR (500 MHz, D_2O), δ 0.95 (3H, t, J=7.3,C**H**$_3$), 1.7 (2H, sext., J=7.3 $CH_3C\mathbf{H}_2CH_2$), 3.1 (2H, t, J=7.3, $CH_3CH_2C\mathbf{H}_2NH$), 3.3 (2H, s, $NHC\mathbf{H}_2CO$), 3.7 (2H, m), 3.85 (1H, dd), 4.0 (2H, m).

EI mass spectrometry revealed a molecular weight of 222 (M^+), which was consistent with previous reports (23).

7.5.10 Coupled assay to determine amadoriase I activity

Assay for determining activity during purification of amadoriase I

The appropriate vols. of reagents outlined in Table 7.25 were added to a cuvette, with the exception of fructosyl propylamine. The cuvette was then incubated in a spectrophotometer blanked to dH_2O in the dark for 5 min at 25°C.

Reagent	Vol (μL)	[Final]
10 mM HEPES, pH 8	825	
Peroxidase (0.5 mg/mL)[a]	40	
ABTS (20 mg/mL)[b]	100	
Test sample	20	
Fructosyl propylamine (32.66 mM)[c]	15	0.49 mM (10x lit K_m) (18)
Total	1000	

Table 7.25: [a] Prepared in HEPES buffer. [b] Prepared in dH_2O. [c] Fructosyl propylamine concentration was determined using the coupled assay and Beer's Law where $A = \varepsilon . c . l$. (ε_{ABTS} at 414 nm, $3.6x10^4$). It was assumed that for every 2 ABTS moles of radical cation molecules generated, 1 mole of fructosyl propylamine is consumed.

Fructosyl propylamine was added to initiate the reaction, whereupon ΔA_{414} was measured for 120 sec. Initial rate was typically calculated from the first 20 sec of the reaction, to ensure that true initial rate was measured, *i.e.* under 10% of the product formed.

A no-substrate control was performed in order to ensure ΔA_{414} was not due to any other reaction components reacting with the enzyme. A no-enzyme control was performed to ensure the ΔA_{414} was solely due to the action of the enzyme.

Experiments to determine K_m *and* V_{max} *of amadoriase I*

It was ensured that enzyme concentration was proportional to rate in the above experiment. This was undertaken as described for the purification of amadoriase I, but with varying enzyme volume (typically a range from 5-50 μL). Compensation for change in volume was made by reducing or increasing the amount of 10 mM HEPES added.

Exactly the same procedure was undertaken for determining the kinetic parameters of Amadoriase I as for purification assays. The only difference being the variation of substrate to final concentrations of 0.005-0.8 mM in the assay. Each concentration was prepared in triplicate, with the experiment undertaken at least in triplicate

Assessment of substrate specificity of amadoriase I

Where possible, substrates of sarcosine oxidase were prepared at 10x literature K_m, or where K_m was not quoted, K_d has been employed. Lower concentrations were due to solubility issues of the compound under study. A standard assay was as outlined in Table 7.26.

Reagent	Vol μL	[Final]
10 mM HEPES, pH 8	Adjusted with differing substrate volumes	
Peroxidase (0.5 mg/mL)[a]	40	
ABTS (20mg/mL)[b]	100	
Test sample	15	
Substrate tested [b]	Varied	1-10 x lit K_m or K_d
Total	1000	

Table 7.26: Volumes required to determine substrate specificity of amadoriase I [a] Prepared in HEPES buffer. [b] Prepared in dH_2O.

Assay conditions and rate calculations were undertaken as stated in previous sections.

7.5.11 Over-expression of amadoriase I mutants

Both mutants, *E. coli* BL21(DE3)pLysS(pET 15bS370A) and *E. coli* BL21(DE3)pLysS(pET 15bH357N) were over-expressed as described in section 7.5.8 for the wild-type enzyme. The crude cell extract of both mutants was tested for activity *via* the coupled assay outlined in section 7.5.10. SDS-PAGE of the crude cell extract was undertaken as outlined in section 7.5.8.

7.5.12 Attempts to restore activity to mildly glycated RNase A

Preparation of RNase A and methylglyoxal incubations (mildly glycated protein)
RNase A was incubated with methylglyoxal as outlined in the Figure 7.1, with incubations at a final concentration of 10 mM methylglyoxal and 25 mg/mL RNase A (1:2 α-dicarbonyl: amine) in 100 mM sodium phosphate buffer (pH 7.4) Appropriate controls were prepared and included as outlined in section 7.2.1 and Figure 7.1 (prepared in duplicate). After preparation of incubations, the quadruplicate samples were halved and two were incubated at 37°C for 1 h whilst amadoriase I (20 μL) or 10 mM sodium phosphate buffer (pH 8) was added to the remaining two which where then immediately frozen in liquid N_2. After incubation of the two initial samples at

37°C, either 20 µL of amadoriase I (120 µg/mL) or 10 mM sodium phosphate buffer (pH 8) was added and the solution incubated at 4°C for 3 days and stored at -80°C at the conclusion of incubation.

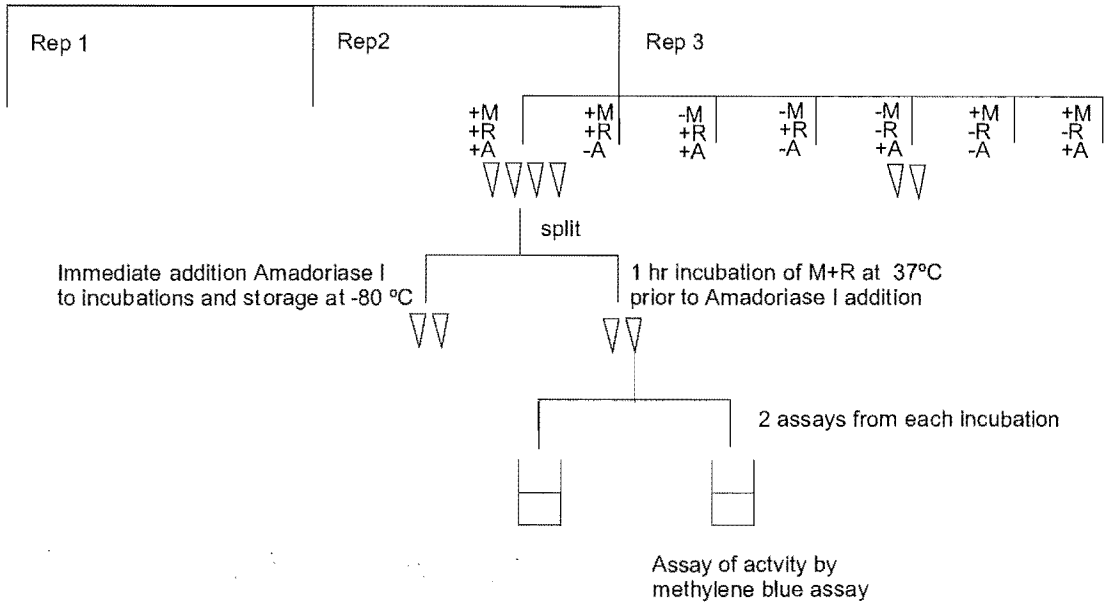


Figure 7.1: Experimental design for testing a mildly glycated substrate. For clarity, only one branch has been extended. Abbreviations: Rep, replicates prepared on different days; M, methylglyoxal; R, RNase A; A, amadoriase I. Controls, -M-R+A, +M-R-A, +M-R+A incubations were only prepared in duplicate, not quadruplicate due to time considerations.

Experiments to determine whether glycated RNase A acts as a substrate for amadoriase I

The experimental procedure was undertaken as outlined in section 7.4. However, a working concentration of 0.06 µg/mL was selected due to batch-wise variation of RNase A. It was ensured that enzyme concentration was proportional to rate exactly as outlined in section 7.4. The methylene blue assay was carried out on all samples, including incubation controls, as outlined for the experiments to determine the catalytic efficiency of RNase A in the presence or absence of Maillard inhibitors (section 7.4).

Statistical analysis

The experiment above was designed as a split plot design with each replicate (Rep) as the block effect. The factors for ANOVA, methylglyoxal, amadoriase and time of incubation were at the plot level. There was a level of nesting where a repeated measurement was taken (of each sample) to measure pipetting error within the assay procedure. Considering the above a multi-way ANOVA was then performed using S-PLUS and the *P* values calculated where required.

7.5.13 Attempts to isolate a potential deglycating enzyme from a Pseudomonad

Preparation of a mildly glycated substrate for bacterial culture

Dialysis tubing preparation

Visking 12,000-14,000 MW cutoff tubing (32 mm x 20 mm, Medicell International Ltd., London, U.K.) was boiled for 10 min in 1 L of 2% (w/v) of sodium bicarbonate and 1 mM $Na_2EDTA{\cdot}2H_2O$. Care was taken to ensure the tubing remained submerged at all times. The tubing was then thoroughly washed in dH_2O and boiled for a further 10 min in 1 L of 1 mM $Na_2EDTA{\cdot}2H_2O$. The tubing was washed in dH_2O and stored, immersed, in 0.1% (w/v) sodium azide. Immediately before use, the tubing was removed from the sodium azide and washed thoroughly with dH_2O.

Casein purification and sugar incubation preparation

Casein (20 g, technical grade) was dissolved in cold dH_2O (4°C, 1 L) and the pH adjusted to 8 using 1 M NaOH. This was dialysed against dH_2O for 3 days (12,000-14,000 MWCO tubing prepared as outlined above) at 4°C with at least 3 changes of dH_2O. The retentate was freeze-dried and resulting casein (13 g yield) was used for subsequent experiments.

A solution of 100 mg/mL lactose (200 µL, prepared in sdH_2O pH 7) was diluted in 200 µL of 50 mg/mL casein (prepared in sdH_2O, pH 7), to give final concentrations of 50 mg/mL lactose and 25 mg/mL casein, then incubated at 37°C for 0, 2 or 5 days. Protein only controls for each time period were prepared as above with the substitution of lactose with sdH_2O. Lactose only controls were also prepared with sdH_2O substituted in place of casein. A day 0 and a day 5 incubation were prepared

for the lactose only controls. Once incubated, samples were dialysed against 1 L of sdH_2O O/N with at least one change of 1 L sdH_2O at 4°C as outlined in section 7.2.3. Samples were stored at -20°C until required.

Protein concentration was quantified by the Bradford method using BSA as a standard as outlined in section 7.5.7. Casein was also employed as a standard with a range of dilutions from 25-250 μg/mL were prepared in triplicate. A working final concentration of 6 μg/mL casein was selected on inspection of the calibration curve.

The decrease of lysine concentration was measured using the *o*PA method outlined in section 7.2.4, using a lysine and a casein calibration curve. A standard solution of 8 mg/mL casein was prepared by dissolving in dH_2O. Dilutions from this were prepared ranging from, 0.5-6 mg/mL in triplicate and assayed as outlined in section 7.2.4. From the calibration curve, a working concentration of 4 mg/mL casein (before dilution in *o*PA) was employed for subsequent assays which were undertaken as section 7.2.4.

Glycated substrate sterilisation procedure

Substrate, the casein and lactose sample, that had been incubated at 37°C for 5 days with subsequent dialysis, was irradiated at 120,000 μJ cm^{-2} for 5 min in glass petri dishes. To confirm sterilisation had been effective, sample (final concentration of 1 mg/mL) was used to inoculate a 3 mL O/N of nutrient broth (Appendix six) and was also spread onto a nutrient agar plate (Appendix six) and incubated at 30°C O/N. No growth in either media deemed the sterilisation procedure effective.

Initial isolation of bacterium of interest

This was undertaken in collaboration with an M.Sc. microbiology student Sarah Day (24).

Purification procedure undertaken to isolate the putative deglycating enzyme (25,26)

1 L of nutrient broth was inoculated with 1 mL of a *P. fluorescens* O/N culture and induced with a final concentration of 1 mg/mL of either sterilized, casein + lactose, casein only or lactose only incubations. Cultures were grown at 30°C for 17 h. A

negative control strain *E. coli* BL21(DE3)pLysS was included, with 1 mg/mL sterilized casein + lactose also added. Each 1 L culture was then cooled to 4°C and centrifuged at 5,000 x *g* for 15 min at 4°C. After centrifugation, the supernatant was carefully removed and discarded and the pellet resuspended in 1x vol of cold (4°C) minimal salts – (3 g monopotassium hydrogen phosphate, 9.17 g dipotassium hydrogen phosphate trihydrate, 0.1 g of ammonium sulfate, 1 mM magnesium sulfate) and respun at 5,000 x *g* for 15 min at 4°C. The supernatant was removed and discarded and the pellet resuspended in 50 mM cold (4°C) sodium phosphate pH 7.4, containing 1 mM $Na_2EDTA{\cdot}2H_2O$ and 0.2 mM phenylmethylsulfonyl fluoride (10 mL/L of culture). Samples were sonicated at 30% amplitude for bursts sonicated for 9 sec on, 9 off for 3 min intervals for a total of 12 min. Cellular debris was removed by centrifugation at 13,000 x *g* at 4°C for 10 min. The supernatant was split and stored at -20 and -80°C. An 8-16% SDS-PAGE gel of the samples was undertaken of the samples (5 μL of crude + 5 μL of 2x treatment buffer) as outlined in section 7.3.2 for RNase A.

Assessment of deglycating ability of crude extract via *amadoriase I assay*

Activity of the crude sample was tested using the assay outlined in Table 7.25 (section 7.5.9), with 20 μL of the crude sample prepared directly above substituted for amadoriase I. 10 μL of 33 mM of fructosyl propylamine (final concentration of 0.49 mM, 10x K_m of Amdoriase I quoted by Wu *et al.*) (18) was added to initiate the reaction. Activity of the crude extract was also measured using a final concentration of 1 mg/mL casein + lactose solution as a substrate in place of fructosyl propylamine.

Assessment of deglycating ability of crude extract via *RNase A methylene blue assay*

A solution of 20 mM methylglyoxal prepared in 100 mM sodium phosphate buffer (pH 7.4) was diluted 1:1 in 50 mg/mL RNase A (in sodium phosphate buffer, pH 7.4) to give a final concentrations of 10 mM and 25 mg/mL respectively (1:2, α-dicarbonyl: amine). Controls were as outlined for similar studies with amadoriase I. Immediately after mixing, an aliquot of M+R and RNase A only control were frozen immediately. The remaining samples were incubated for 1.5 h and then half of the M+R samples and half of the protein only controls were incubated with 10 μL of the crude *Pseudomonas* that had been cultured on casein + lactose and stored at 4°C for

19 h. The remaining samples were incubated with 10 μL of 50 mM sodium phosphate pH 7.4 containing 1mM $Na_2EDTA{\cdot}2H_2O$ and 0.2 mM phenylmethylsulfonyl fluoride, in place of crude extract and incubated for the same period of time as those containing the crude extract.

Samples were then tested for activity using the RNase A assay outlined in section 7.5.10. The following was dispensed into a cuvette with the exception of the RNase A ± methylglyoxal incubation (Table 7.27).

Reagent	Vol. (μL)	[Final] (μg/mL) (2 s.f.)
RNA (10 mg/mL)	60	600
Methylene blue buffer	940	
Sample - RNase A (6 μg/mL, in MOPS buffer) ± dicarbonyl ± *Pseudomonas* extract	10	0.06

Table 7.27: Vols. required to estimate the deglycating ability of the Pseudomonas extract

The remainder of the assay was undertaken as outlined in section 7.4.1.

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Appendix one

p-Hydroxybenzoic acid hydrazide (HBH) method 1 quantify α-dicarbonyl concentration

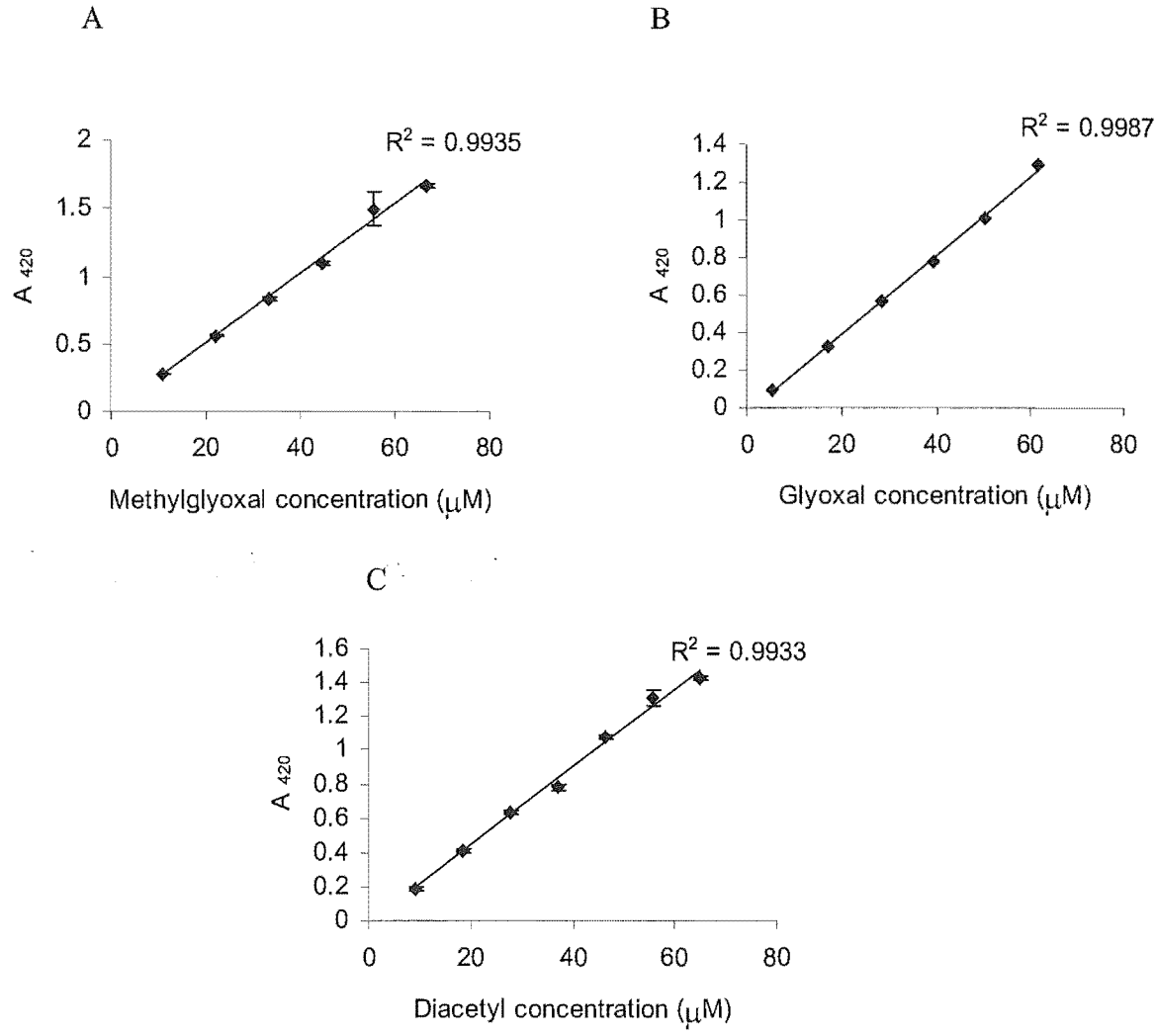


Figure 1: Calibration curves for α-dicarbonyls A, methylglyoxal; B, glyoxal; C, diacetyl. Concentrations above are initial concentrations prior to dilution in HBH. Readings for each time point represents an average of three samples. Error is expressed as standard error of the mean.

The first HBH method attempted to quantify α-dicarbonyl decrease in incubations that contained RNase A and the results are detailed in Figure 2.

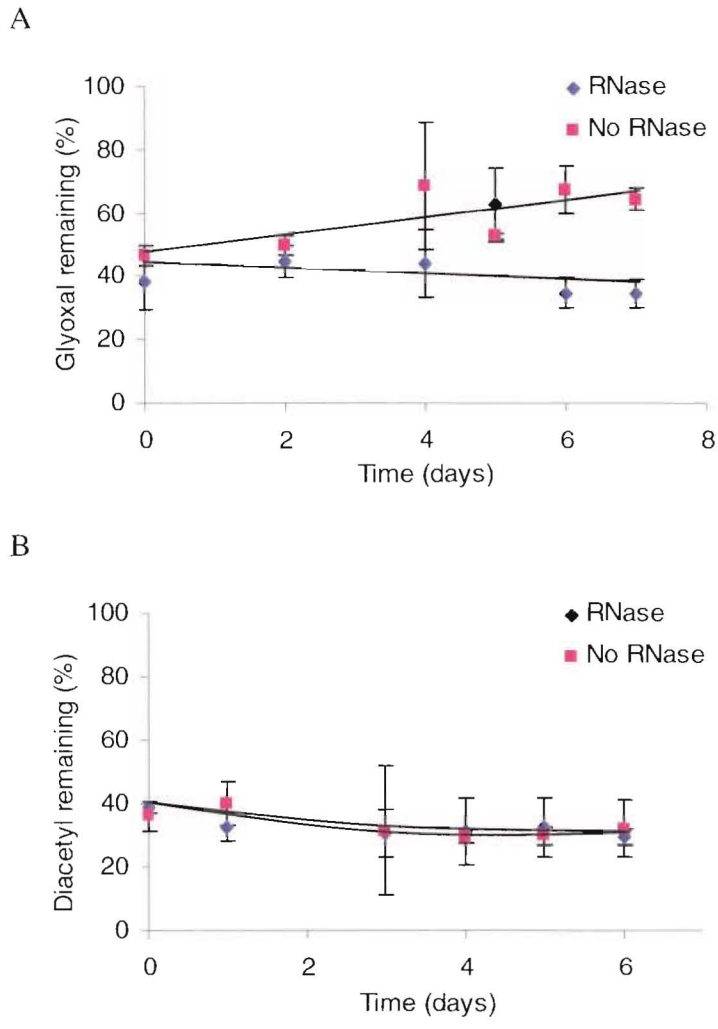
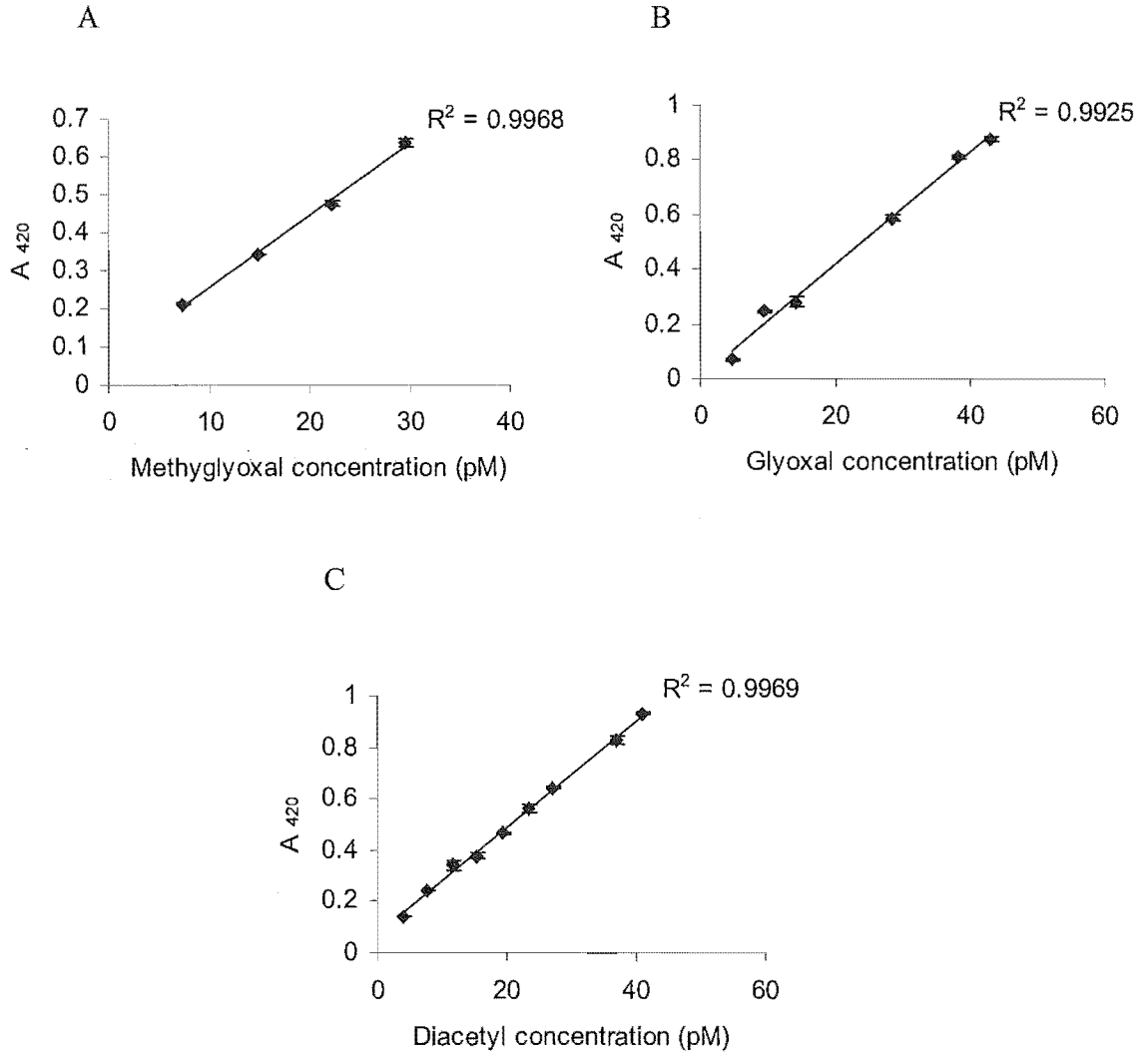


Figure 2: A measure of α-dicarbonyl concentration over time for A, glyoxal; B, diacetyl; in presence or absence of RNase A. Readings for each time point represents an average of three samples. Error is expressed as standard error of the mean.

p-Hydroxybenzoic acid hydrazide (HBH) method 2 to quantify α-dicarbonyl concentration

Calibration curves were constructed for all three α-dicarbonyls and are outlined in Figure 3.



C

Figure 3: Standard curves for α-dicarbonyls A, methylglyoxal; B, glyoxal; C, diacetyl Concentrations above are initial concentrations before dilution in HBH. Readings for each time point represents an average of three samples. Error is expressed as standard error of the mean.

The results of an attempt to quantify glyoxal decrease in incubations that contained RNase A are detailed in Figure 4. Methylglyoxal was trialled but very large errors were obtained.

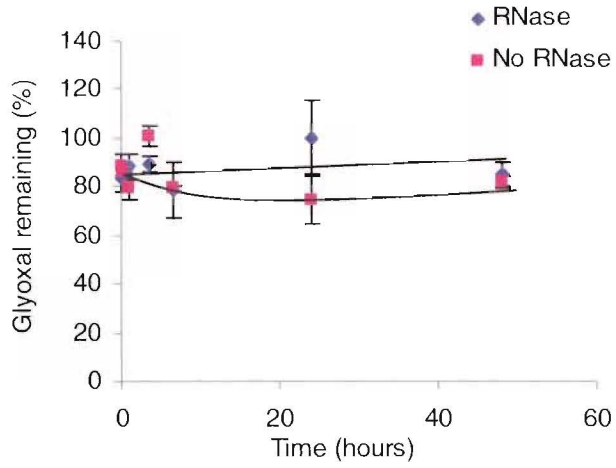


Figure 4: Decrease in glyoxal over time in the presence or absence of RNase A in incubations. Readings for each time points are an average of three samples. Error is expressed as standard error for the mean for triplicate samples.

The effect of phosphate on the crosslinking reaction

The effect of phosphate on the rate of the Maillard reaction in RNase A containing methylglyoxal was assessed by the *o*PA assay and the data subjected to an analysis of variance (ANOVA). The data is represented in Table 1. Along with the effect of phosphate, the time of incubation was also taken into consideration, but did not have an additive effect to the decrease in lysine concentration. This may be due to the break-down of RNase A over time as discussed in chapter 2 (measurements of time ranged from 0 – 48 hours). "Preparation" and "Sub" were duplicate incubations prepared from the same stock solution, in order to account for pipetting error within the experiment. These are referred to as repeated measurements, or, nesting. Another set of repeated measurements at the residual level was performed during the assay itself, to detect any discrepancies within the assay.

Treatment	df	deviance	MS	F	*P*
Phosphate	1	5.66	5.66	75.34	0.01
Time	6	0.12	0.02	0.77	n.s.
Phosphate:Preparation	2	0.15	0.08	2.05	n.s.
Time: Phosphate	6	0.08	0.01	0.47	n.s.
Phosphate: Preparation:Sub	4	0.15	0.04		
Time:Phosphate:Preparation:Sub	36	0.97	0.03	1.55	n.s.
Residual	43	0.75	0.02		
Total	98	7.9			

Table 1: Multi-way ANOVA of a split-plot design with 2 series of repeated measurements (nesting) at the subpreparation (Sub) and the residual level. n.s, non-significance, was deemed to be $P>0.05$.

Appendix two

Supplemental graphs and gels for experiments detailed in Chapter four

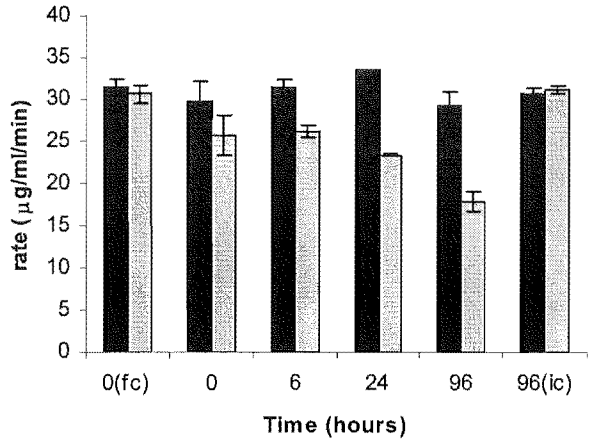


Figure 1: RNase A activity over 96 hours following incubation with glyoxal in the presence (■), or absence ( ) of aminoguanidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate reading. Abbreviations are fc, frozen control, ic, incubated control, 0-96 h incubation time of α-dicarbonyl-containing RNase A samples.

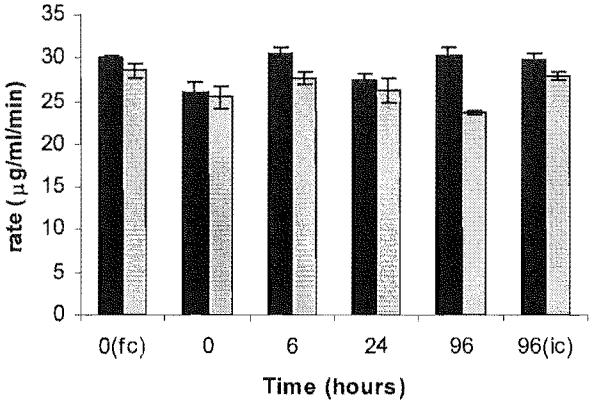


Figure 2: RNase A activity over 96 hours following incubation with diacetyl in the presence (■), or absence ( ) of aminoguanidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate reading. Abbreviations are as for Figure 1.

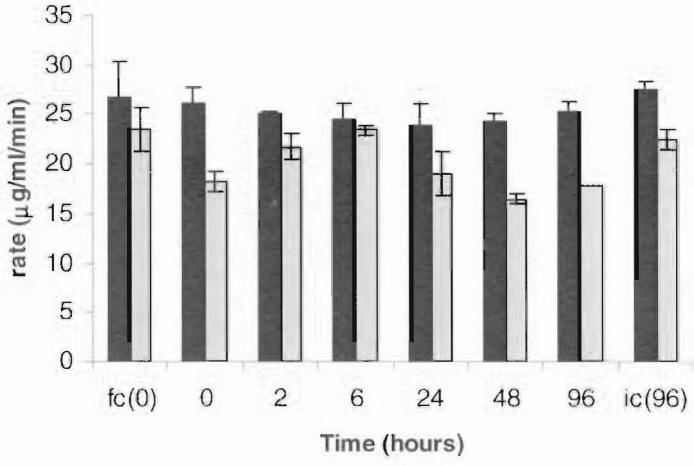


Figure 3: RNase A activity over 96 hours following incubation with glyoxal in the presence (■), or absence ( ), of 3,5-dimethylpyrazole-1-carboxamidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate reading. Abbreviations are as for Figure 1.

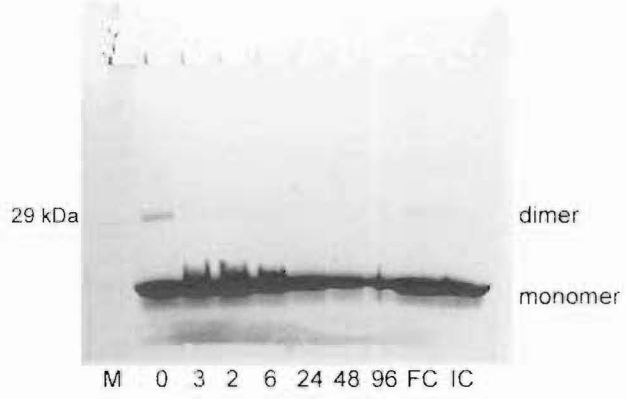


Figure 4: 8-16% SDS-PAGE gel RNase A incubated with glyoxal and DMPC Abbreviations: M, marker, 0-96 time of incubation of α-dicarbonyl-containing RNase A sample in the presence of inhibitor, FC, frozen control, IC incubated control.

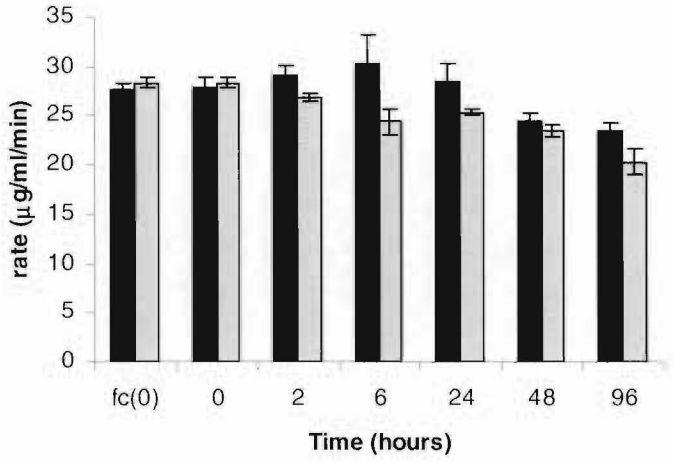


Figure 5: RNase A activity over 96 hours following incubation with diacetyl in the presence (■), or absence ( ) of 3,5-dimethylpyrazole-1-carboxamidine. Bars indicate the average of data generated from triplicate readings. Error bars represent the standard error of the mean for the triplicate reading. Abbreviations are as for Figure 1.

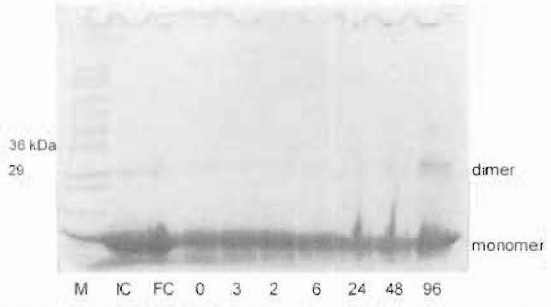


Figure 6: 8-16% SDS-PAGE gels RNase A incubated with diacetyl and DMPC Abbreviations are as for Figure 4.

Appendix three

Statistical analysis of data from experiments undertaken in Chapter five to assess whether amadoriase I restored glycated RNase A to its native function

Treatment	df	deviance	MS	F	P
Day	3	892.98	297.66	11.57	0.003
Amadoriase	1	90.87	90.87	3.53	n.s.
Sugar	1	161.08	161.08	6.26	0.04
Time	1	8.66	8.66	0.34	n.s.
Amadoriase:Sugar	1	5.30	5.30	0.21	n.s.
Amadoriase:Time	1	14.26	14.26	0.55	n.s.
Sugar:Time	1	444.47	444.47	17.27	0.003
Amadoriase: Sugar: Time	1	10.41	10.41	0.40	n.s.
Amadoriase: Sugar: Time: Subprep	8	205.88	25.73	1.19	n.s.
Residual	97	2099.83	21.65		
Total	115	3933.744	1080.097		

Table 1: Multi-way ANOVA with one level of nesting at the time level. n.s., non-significance, was deemed to be P>0.05.

Each experiment was repeated in quadruplicate on different days (daywise variation was assessed in Table 1 by "Day"). As predicted, incubation of RNase A and sugar over time showed a significant difference in activity over those that did not contain sugar, as measured by the Sugar:Time interaction term.

References

1. Zarr, J. H. (1996) *Biostatistical analysis*, 3rd Ed., Prentice-Hall International, New Jersey

Appendix four

Site-directed mutagenesis of amadoriase I

Little is known about the amino acids that are involved in the catalytic mechanism of amadoriase I. This section aimed to address this issue with two mutations to the putative active site. The mutations described here were made in collaboration with Andrea Uhlmann (His357 to Asn) and Stephan Hegge (Ser370 to Ala mutation), during exchange projects from Germany. Initial BLAST and literature searches for residues of amadoriase I relevant to substrate binding and catalysis were undertaken by myself. Both students were responsible for final selection of which residue to mutate, primer design and practical work, which I oversaw.

Conserved residues within the amadoriases

Figure 1 outlines sequence alignment data for all amadoriases isolated to date along with bacterial sarcosine oxidase mentioned previously. The region ranging from residues 340-400 in amadoriase I is highly conserved with respect to other amadoriases. This is thought to contain the flavin binding site where the conserved Cys residue for flavin binding resides (in the case of Amadoriase I this is Cys342) and also residues that may be important in catalysis. Two residues of particular interest were serine 370 and histidine 357, which are conserved within all of the amadoriases (Figure 1). These residues were mutated to alanine and asparagines respectively to probe their role in the catalytic mechanism of amadoriase I, with the schemes proposed in section 5.3.1 in mind.

Figure 1: A highly conserved region of Amadoriases thought to contain the active site of the amadoriases. The area shown here is from Cys342 (in Amadoriase I) to Gly 381. Black blocks denote 100% identity, grey blocks denote 75%-99% identity. + denotes a conserved Cys, that is thought to be the point of FAD covalent attachment. * denotes residues that have been mutated.

Creation of the amadoriase I mutants

The primers for both mutants, Ser 370 to Ala (S370A) and His 357 to Asn (H357N), are outlined in Table 1. Both mutants were generated by site-directed mutagenesis using the Stratagene QuikChange Mutagenesis Kit, according to the manufacturer's instructions (1). Sequencing was undertaken using T7 promoter and T7 terminator primers, along with a custom primer that bound to DNA in the1002 and 1025 bp region of amadoriase I (mutations at 1115 and 1154 bp) to obtain a complete read-through of the amadoriase I sequence.

	Primer sequence
WT	5' GCT GTT GGA GGT TCC GGC AAT GGC GCC 3'
S370A (#1)	5' GCT GTT GGA GGT **G**CC GGC AAT GGC GCC 3'
S370A (#2)	5' GGC GCC ATT GCC GG**C** ACC TCC AAC AGC 3'
WT	5' C CGT GCT TTC TTG ATC GAT AGA CAT CCT GAA CAC CCC 3'
H357N (#1)	5' C CGT GCT TTC TTG ATC GAT AGA **A**AT CCT GAA CAC CCC 3'
H357N (#2)	5' GGG GTG TTC AGG A**T**T TCT ATC GAT CAA GAA AGC ACG G 3'

Table 1: Primers used for site-directed mutagenesis experiments. WT denotes wild type sequence for the area of interest. #1 represents the sense primer. #2 represents the antisense primer. The codons of interest are underlined and the bases to be changed, are depicted in bold.

Following successful amplification of each mutant plasmid, *E. coli* XL1-Blue was successfully transformed with this DNA. In the case of the S370A mutant, the plasmid DNA was prepared and examined by restriction analysis to confirm the appropriate mutation had been made. This was made possible by the creation of a new restriction site, recognized by *Nae*I as a result of the substitution of T for G (Figure 2). The results of the digest are shown in Figure 3. Bands with the sizes of 3.4, 1.6, 1.3, 0.4, 0.35 and 0.16 kb were expected, with bands at 3.5, 1.5, 1.3, 0.39, 0.29 and 0.16 observed (Figure 3).

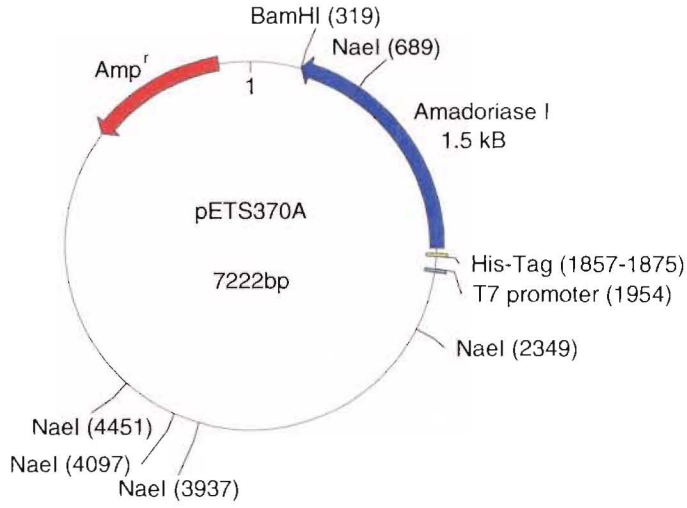


Figure 2: pET 15b vector map containing the amadoriase I insert with Ser 370 substituted with Ala. Vector map was created using DNAMAN (Lynnon Corporation, Quebec, Canada). The numbers in brackets denote the base number.

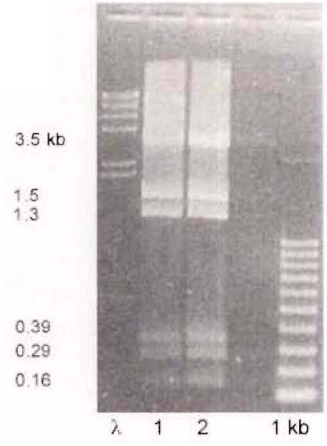


Figure 3: An agarose gel of a restriction analysis of mutant S370A plasmid DNA. From left to right: λ cut with *Hin*dIII (see Figure 5.8 for marker molecular weight); 1 and 2, pETS370A cut with *Bam*HI and *Nae*I; 1kb marker, molecular weights from top to bottom, 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 kb.

Unfortunately, creation of a new restriction site was not simple with the H357N mutant, and the mutation was confirmed by sequence analysis at the Waikato sequencing facility. Subsequent to confirmation of both mutations, the expression strain, *E. coli* BL21(DE3)pLysS was transformed with the mutant plasmids.

Purification and kinetic assay of S370A and H357N mutants

Both strains containing each mutation were cultured and purified as outlined for the wild-type enzyme. Assessment of activity involved the use of the coupled assay described previously. Interestingly, the crude extract of the S370A mutant did not show any activity, yet SDS-PAGE analysis confirmed the over-expression of a protein at 50 kDa that corresponded to the molecular weight of amadoriase I. Work thus far suggests that that both mutagenesis experiments have been successful and two inactive mutants of amadoriase I have been created.

The preliminary results reported here are encouraging, as both mutations have resulted in a total loss of amadoriase activity. However, further experiments must be undertaken to assess whether these enzymes are critical for catalysis, or the mutation has resulted in the formation of a misfolded protein. Future experiments involving circular dichroism will seek to test the latter point.

References

1. Stratagene (2002) *QuikChange mutagenesis kit instruction manual*, http://www.stratagene.com/manuals/200518.pdf

Appendix five

The search for novel deglycating enzymes

Amadoriase I cannot act on glycated protein, thus, from a therapeutic standpoint, it has limited potential. The search for a novel enzyme that can use glycated protein as a substrate has become a focal point for research (1). This section outlines work, initiated by Sarah Day (Microbiology M.Sc. student working with Dr. John Klena, University of Canterbury), aimed to isolate a potential deglycating enzyme (2). In the past, micro-organisms that metabolise glycated model amines have generally been isolated from soil samples (3). Here, a different environment has been studied, raw milk. Milk is rich in casein and sugars, such as lactose, that participate in the Maillard reaction (4,5). The glycated substrate for bacterial isolation experiments, and assessment of the activity of the successful isolate toward glycated protein was undertaken by myself. Sarah Day was responsible for the culturing, isolation and identification of the bacteria from raw milk that were found to grow successfully on a glycated protein substrate compared to protein only or sugar only controls (2).

Selection and generation of a suitable substrate

Casein is an excellent candidate for *in vitro* glycation experiments, as it is present in milk and commercially available at little expense. Additionally, this protein is reported to be extremely heat stable (6), and as a result, has been intensively studied by those Maillard chemists working in the food industry. Casein comprises 80% of the milk proteins which constitutes 3.3% of milk (5), and is divided into four groups, α_{s1}-, α_{s2}-, β- and κ-casein. Casein exists in large colloidal micelles with calcium phosphate and has almost no secondary or tertiary structure (5,6). Maillard-type reactions between casein and lactose have been found to result in the gelling of milk (5). A decrease in lysine concentration and concomitant increase in AGEs have been reported in casein on treatment with aldoses, ketoses and α-dicarbonyls. (4,7) Melanoidin pigments, formed in the later stages of the Maillard reaction have been reported also in sugar-casein systems (6).

Lactose (Figure 1) is a disaccharide that is the predominant sugar in milk, comprising 5% of the content of milk (5). The sugar has been studied in Maillard systems where it has been found to modify β-lactoglobulin, another milk protein (8).

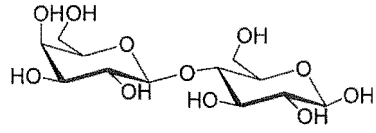


Figure 1: The structure of lactose: galactose and glucose monomer linked by a β 1-4 glycosidic linkage.

On reaction of lactose with lysine, the Amadori product, lactuloselysine, can be formed following Amadori rearrangement (Reviewed in (9)). The structure of this compound, which has been used as a marker of early glycation in milk, is outlined in Figure 2. Interestingly, CML, introduced in Chapter one, is isolated from human tissue, can be formed from this compound (Reviewed in (9)).

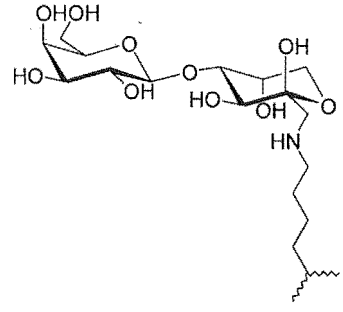


Figure 2: The structure of lactuloselysine (9).

Generation of the glycated substrate protein involved the incubation of casein with either lactose or xylose. Lactose was selected due to is predominance in milk, making it a most likely candidate for modifying residues within casein. Thus, if a deglycating enzyme did exist, in milk, it would most likely recognize this form of glycated protein. Xylose was selected due to its mild glycating ability and the fact that its role in the Maillard reaction is reasonably well-studied (10,11).

The extent of the Maillard reaction was measured by the decrease in lysine concentration as determined by using the *o*PA method. Calibration curves for lysine and casein were both prepared (Figures 3 A and B).

A B

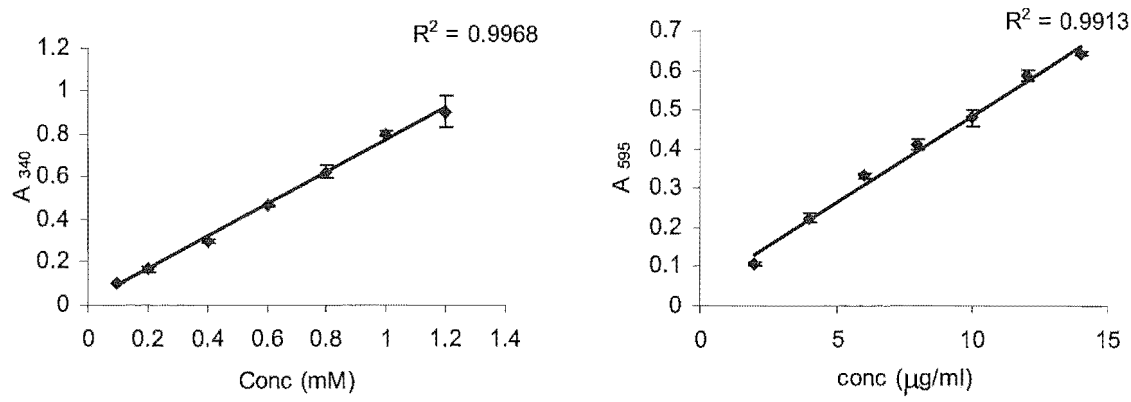


Figure 3: Calibration curves of A, lysine and B, casein to measure lysine content *via* the *o*PA method and protein concentration *via* the Bradford assay respectively. Each point is an average of triplicate concentration samples. Error is expressed as standard error of the mean of triplicate samples.

Following inspection of the calibration curves, the appropriate assay concentrations were selected. The decrease in lysine concentration in casein in the presence of either xylose (Figure 4) or lactose (Figure 5) was then measured. In these experiments, a protein only control was included for each time point incubation, and used as a reference for calculation of lysine percentage. Initial work with xylose was not extended due to the fact that xylose can reversibly modify protein (12), and thus could cause false positive results. After extensive testing, the conditions of 50 mg/mL lactose and 25 mg/mL casein incubated for 2 and 5 days at 37°C were selected for ensuing experiments.

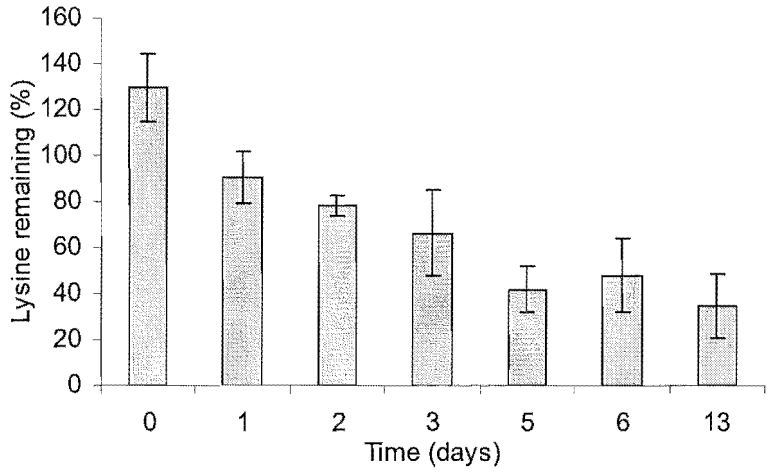


Figure 4: Percentage lysine remaining in casein following incubation with xylose. Readings are expressed as percentages of the protein only control prepared for each appropriate time point and are an average of triplicate experiments. Error is expressed as standard error of the mean for triplicate experiments.

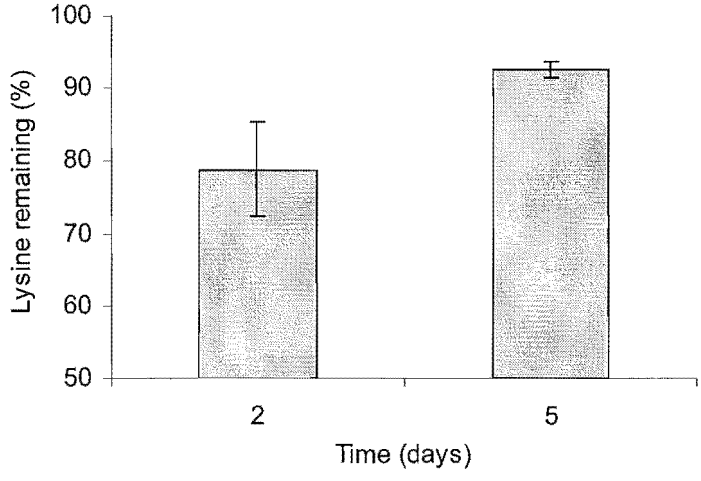


Figure 5: Percentage lysine remaining in casein on incubation with lactose. Readings are expressed as percentages of the protein only control prepared for each appropriate time point are an average of duplicate experiments. Error is expressed as standard error of the mean for duplicate experiments.

The increase in lysine residues from day two to day five (Figure 5) may be due to the break-down of casein during incubation with the lactose. This increase of lysine over time was also noted for RNase A in chapter two.

A significant result from the work undertaken by Sarah Day, was the isolation of a bacterium, *Pseudomonas fluorescens*, that exhibited the capacity to grow more efficiently on glycated protein substrate than protein or lactose alone (2). When supplemented with casein only, this bacterium was shown to commence growth after 72 h. However, when supplemented with the glycated substrate, growth was almost immediate. The difference in growth was the most apparent using a 25 mg/mL casein + 50 mg/mL lactose solution that had been incubated for 5 days at 37°C.

The next phase of this research, after finding a promising bacterial candidate, was to attempt to isolate the enzyme(s) that enabled the bacterium to grow more efficiently on the glycated substrate as opposed to the controls. The above incubation conditions for the preparation of the glycated substrate were employed for all subsequent experiments to isolate the enzyme that allowed for the rapid growth of *P. fluorescens* on a glycated substrate. Initial work to create a glycated substrate involved tracking the progress of the reaction by lysine analysis. After two days of incubation with lactose at 37°C, 74% of lysine remained available within casein. After 5 days however, it appears that this is raised to around 90%. However, this may be due to issues encountered in Chapter two where the breakup of the protein over time overshadows perceptible lysine loss.

Isolation of an "amadoriase-like" enzyme from P. fluorescens

In order to isolate the putative enzyme, *P. fluorescens* was cultured in the presence of glycated substrate. It is essential to include the substrate during the growth of the bacterium, as the expression of some amadoriases is induced by the glycated substrate (13). To ensure the absence of contaminating bacteria within the substrate, samples were UV-irradiated prior to use. The period of exposure was kept to a minimum to ensure that protein structure was not disrupted but bacterial contaminants were eliminated. This was also undertaken in initial experiments undertaken to isolate deglycating bacteria (2). Attempts to use less harsh methods of sterilization such as filter sterilization resulted in difficulties (2). The purification protocol adopted was that outlined by Gerhardinger *et al.* and Saxena *et al.* who isolated proteins from a Pseudomonad that could either bind glycated low molecular weight amine or enzymatically cleave glycated low molecular weight amine (14-16).

Testing the cell extract for "amadoriase-like" activity

Once the cells were harvested using methods described by Gerhardinger *et al.* and Saxena *et al.*, the crude sample was tested for activity using two approaches. The first involved the coupled ABTS assay that had been used to determine the kinetic parameters of amadoriase I earlier in this chapter (section 5.4.2), with the crude extract in place of amadoriase I. The first attempt used fructosyl propylamine, the substrate that had been used for the kinetic study of amadoriase I, but gave a negative result. It was thought that perhaps as the bacterium had always been cultured using glycated protein as a substrate that the enzyme may not recognize glycated low molecular weight amines. Thus, glycated protein was substituted for fructosyl propylamine in the coupled assay. This again resulted in negative readings, which may be due to the fact that this enzyme does not produce hydrogen peroxide. This has been reported to be the case for one deglycating enzyme isolated from a Pseudomonad (16).

A second approach was undertaken to circumvent difficulties noted using the coupled assay, as outlined in the paragraph above. The crude mixture was assessed for its ability to deglycate RNase A, and thus effect a return to function, which could be measured by the RNase A activity assay (section 5.7). This experiment was trialed using the mildly glycated protein that had been used in section 5.7.1 for the amadoriase I. Briefly, a 10 mM methylglyoxal, 25 mg/mL RNase A solution was incubated at 37°C for 1 hour. After this the crude extract was added and incubated overnight at 4°C. The experiment was undertaken exactly as for the amadoriase work and a negative result was obtained (Figure 6).

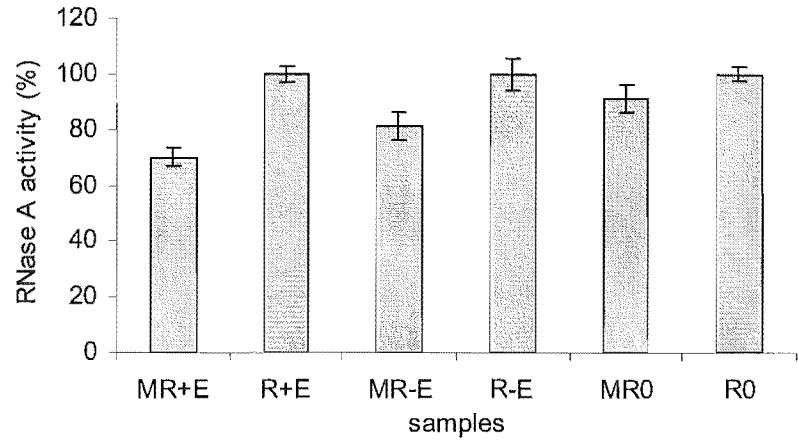


Figure 6: Attempts to return activity from glycated RNase A by the *P. fluorescens* crude extract. Abbreviations, MR+E, methylglyoxal + RNase incubated for 1 hour followed by addition of *Pseudomonas* extract and incubation overnight at 4°C; R+E, RNase incubated for 1 hour followed by addition of *Pseudomonas* extract and incubation overnight at 4°C; MR-E, methylglyoxal + RNase incubated for 1 hour followed by addition of 50 mM sodium phosphate buffer (containing 1 mM EDTA and 0.2 mM PMSF) and incubated overnight at 4°C; R-E, RNase incubated for 1 hour followed by addition of 50 mM sodium phosphate buffer (containing 1 mM EDTA and 0.2 mM PMSF) and incubated overnight at 4°C; MR0, methylglyoxal + RNase A frozen immediately, R0, RNase A frozen immediately. Readings are an average of duplicate experiments and are expressed as a control for the appropriate RNase control. Error is expressed as standard error of the mean for duplicate experiments.

On inspection of Figure 6 it is evident that RNase A activity is lowered in the methylglyoxal and RNase A incubations that had been treated with the crude extract. Although RNase A is a stable protein and a serine protease inhibitor in the form of PMSF, has been added to the crude sample, either break-up of the protein is occurring or inhibitors are binding to RNase A to lower activity. The crude extract does not appear to aid the return to function of the glycated protein that was hypothesised.

Although attempts were unsuccessful to isolate a deglycating enzyme from milk isolates it must be considered that there may be a protein that can bind the glycated substrate in the crude mixture, but not cleave, as has been noted elsewhere in experiments with *Pseudomonas* (14).

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Appendix six

Strains, media and solutions required for molecular biology and protein expression in Chapter five.

Strain list

Strain	Genotype
E. coli BL21(DE3)pLysS	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ(DE3) [pLysS Cam^r]
E. coli BL21(DE3)pLysS (pET 15b)	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ(DE3) [pLysS Cam^r] [pET 15b::*amaI* Amp^r]
E. coli BL21(DE3)pLysS (pET 15bS370A)	*E. coli* B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ(DE3) [pLysS Cam^r] [pET 15b::*amaI*S370A Amp^r]
E. coli BL21(DE3)pLysS (pET 15bH357N)	B F$^-$ *dcm ompT hsdS*(r_B-m_B-) *gal* λ(DE3) [pLysS Cam^r] [pET 15b::*amaI*H357NAmp^r]
E. coli XL1-Blue	*recA1 endA1* gyrA96 *thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}ZΔ*M15* Tn10 (Tet^r)]
E. coli XL1-Blue (pET 15b)	*recA1 endA1 gyrA96 thi*-1 *hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}ZΔ*M15* Tn10 (Tet^r)] [pET 15b *amaI* Amp^r]
E. coli XL1-Blue (pET 15bS370A)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}ZΔ*M15* Tn10 (Tet^r)] [pET 15b *amaI*S370A Amp^r]
E. coli XL1-Blue (pET 15bH357N)	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}ZΔ*M15* Tn10 (Tet^r)] [pET 15b *amaI*H357N Amp^r]

LB-Agar plates

20 g of LB and 15 g of agar were added to 1 L of dH_2O, mixed and autoclaved before use. On cooling to 50°C, antibiotic was added where required, and the LB-Agar was poured into Petri dishes and the surface of the molten agar flamed to removed bubbles. Plated were then dried for 10 min in a biohazard hood.

NA-Agar plates

13 g of nutrient broth and 20 g of agar were added to 1 L of dH_2O, mixed and autoclaved prior to use. On cooling, media was poured into Petri dishes and the surface of the molten agar flamed to removed bubbles. Plates were then dried for 10 min in a biohazard hood.

LB for liquid culture

20g LB was added to 1 L of dH_2O, mixed and autoclaved before use.

Nutrient broth for liquid culture

13g of nutrient broth was added to 1 L of dH_2O, mixed and autoclaved before use.

Preparation of antibiotics and IPTG for bacterial selection and induction

All stocks were prepared at 1000x concentration

Chloroamphenicol: 30 mg/mL dissolved in 100% EtOH, stored at -20°C until required.

Ampicillin: 100 mg/mL dissolved in sdH_2O, filter-sterilised and stored at -20°C until required.

Tetracyline: 20 mg/mL dissolved in 100% EtOH and stored at -20°C until required.

250 mM IPTG stock: 297.9 mg IPTG in 5 mL of sdH_2O, filter-sterilized stored at -20°C.

SOC media for electroporation experiments

2% w/v bactotryptone
0.5% 2/v yeast extract
10 mM sodium chloride
2.5 mM potassium chloride
10 mM magnesium chloride
10 mM magnesium sulfate

0.36% w/v glucose

prepared in dH_2O and autoclaved prior to use.

Preparation of X-gal plates for site-directed mutagenesis experiments

100 µL of 10 mM IPTG (prepared in dH_2O, filter-sterilised) and 100 µL of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, prepared in DMF) was spread onto LB-Agar plates containing the appropriate antibiotics and then dried in a biohazard hood to remove DMF. This was undertaken 30 min prior to plating out transformants.

NZY+ broth for transformation of XL1-Blue with Amadoriase I mutants plasmids

NZ amine (10 g), yeast extract (5 g), and NaCl (5 g) was added to ddH_2O (1 L). The pH was adjusted to 7 with the addition of NaOH before the media was sterilised by autoclave. Following this, $MgCl_2$ (1 M, 12.5 mL), $MgSO_4$ (1 M, 12.5 mL), and glucose (20%, 20 mL) were added (all were filter-sterilised prior to addition).